CYTOLOGICAL STUDIES ON CELLULAR DEGENERATION OF DIFFERENTIATED AND UNDIFFERENTIATED TISSUES IN VITRO[1]

by

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INTRODUCTION.

The early techniques of tissue culture, whereby tissues and cells are allowed to proliferate in cultures for a brief period at the expense of their own nutriment or without sub-culturing, have been so strongly criticized that degenerative phenomena of any kind are generally most sedulously avoided in recent cytological work. Every attempt has been made of late to work with cultures of cells showing undiminished proliferation and unchanging morphology, throughout an indefinite number of sub-cultures.

When a concise description of the cytological changes involved in cytolysis was sought for, it was found that the present literature on the degeneration of tissues and cells gave a very incomplete and conflicting survey of the process.

In fact, degenerative phenomena were almost invariably the subject of chance observations recorded at random in various cytological and pathological communications.

It is not surprising that many of these scattered observations are conflicting in nature. In pathology cytolysis is necessarily frequently observed, but the rather crude methods of fixation, so commonly used, make any detailed cytological examination difficult. Moreover, stained sections of tissue give a poor picture of the morphology of the individual cellular components, whereas their isolation *in vitro* cultures preserves all their characteristics with great constancy. To obtain any complete idea of the process of cytolysis, it is necessary to ma. observations at consecutive intervals upon the living cells, as well as using stained cultures of the same types of cells fixed at different stages of degeneration.

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This investigation has been undertaken to give a concise description and to help to elucidate the phenomenon of cytolysis, and to correlate and discuss the scattered data already recorded by previous investigators.

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MATERIAL AND METHODS OF TISSUE CULTIVATION.

Throughout this investigation embryonic tissues from two and seven days' old chick embryos were cultivated *in vitro* by the usual hanging-drop technique Fowl plasma was used as a supporting medium and embryonic extract as a nutrient medium.

Preparation of Extracts: The chick plasma, which was obtained from the ventricle of adolescent fowls, was collected by means of dry sterile canulae, into paraffin-coated Wasserman tubes previously cooled to 0° C. The supernatant fluid, obtained by centrifuging, was employed as the supporting medium, and stored in paraffined tubes within the refrigerator. The nutrient extract was prepared by the mincing of seven-day chick embryos in a given volume of saline solution of 7·5 pH. The mixture was finally centrifuged; the supernatant fluid, after pipetting off, was then employed as the nutrient medium.

Differentiated tissues selected for *in vitro* cultivation were obtained from the heart of seven-day chick embryos, and somatic mesoderm was isolated from 48-hour chick embryos and employed in the observations on undifferentiated tissues.

Before implantation the selected portions of embryonic tissues were washed in a sterile saline solution of 7·5 pH. The isolated fragments were then placed upon a No. 1 cover-slip, and after equal volumes of embryonic extract and plasma were added the fluids were mixed, and the whole inverted over a hollow ground slide, sealed in the usual manner, and incubated at 38·5° C.

In order to incur cytolysis the cultures were allowed to remain in an unchanged medium for periods ranging up to nine days. As the cytotoxins accumulated in the medium, the explants gradually underwent degenerative changes. In some cases this process was accelerated by subjecting the cultures to a temperature of 0° C.

The cultures were fixed, after various intervals of degeneration by osmic vapour, and were stained by the Heidenhain iron haematoxylin method. To carry out investigations *in vivo* the implants were placed upon a warm stage maintained at body temperature. In order to depict the structure and behaviour of the living, unstained, degenerating cells, microphotographs were taken at various intervals of cytolysis by direct illumination. For a study of the cyto-microsomes, cultures were stained *intra vitam* with Janus green B and neutral red.

DEGENERATIVE PHENOMENA AS SEEN IN DIFFERENTIATED AND UNDIFFERENTIATED TISSUES *IN VITRO*.

After several hours' incubation *in vitro*, cells may be seen proliferating in a radial manner and migrating from the explanted tissue into the surrounding medium. An examination, after 24 hours' cultivation, will show that this area of new growth, which has penetrated out farther into the culture medium, has the appearance of a thin sheet, which generally adheres to the under-surface of the cover-slip. In living cultures, fixed and stained after 24 hours' incubation, this area of new growth presents an admirable field for cytological investigation.

A study of the isolated, individual, cellular elements of the explant at this period of incubation reveals the cells in a healthy, normal condition, as the unchanged medium is as yet unaffected by the accumulation of toxins.

Before describing in detail the cytological changes which occur during cytolysis in differentiated tissues, the structure and appearance of the normal healthy cell will be depicted, and for this purpose isolated fibroblasts forming the periphery of a 24 hours' culture were chosen as controls. The external structure of a healthy fibroblast is dependent largely as to whether the cell happens to be growing upon the under-surface of the cover-slip or in the medium adjacent to the cover-glass. When observed in the former position they appear in a triangular or polyhedral form, while those growing in the latter manner possess a spindle-shaped structure.

The inner cytological structure of a healthy fibroblast as seen after fixation is of interest (see figure 1). The nucleus, which is generally situated within the centre of the cell, possesses a characteristic oval shape, a diffuse chromatin network, as well as a clear nuclear membrane. A large nucleolus or plasmasome is invariably present in all well-stained cells. The variable number of cyto-microsomes, arranged within the somewhat opaque protoplasm, presents an interesting field. Mitochondria, lipoidal droplets, together with other cellular structures, some having the appearance of the Golgi reticulum, are all typical features of the healthy cell, and the structure and appearance of these bodies are all indicative of a normal metabolic process.

That quantitative changes in the form of an increase in number of the cell-mitochondria and other cytoplasmic inclusions are constantly met with in pathological conditions, has been demonstrated by Homans (11) and Policard (31). It is also significant that mitochondria are sensitive to cell injury, and it has been shown in many cases that injury to the cell produces a change in the form as well as an increase in the number of these bodies.

The first evidences of cytolysis may be detected in cultures that have been cultivated *in vitro* for about 50 hours in an unchanged medium. As the cyto-toxic products of the medium accumulate, due to prolonged cultivation without sub-culturing, gradual changes in the intracellular bodies, as well as in the

general contour of the cells, are first observed. Instead of the almost triangular morphology of the healthy fibroblast, the cell becomes more elongated and irregular in outline. Needle-like processes, in the form of fine pseudopodia, are invariably sent out from the periphery of the cell (see figure 2).

Frequently, during this early stage of cytolysis, an interesting process of an unequal division of the cell protoplasm occurs, whereby a small portion of the cytoplasm becomes constricted from the main body of the cell, swells up, and remains attached for some time, without nuclear material, by a very slender, thread-like pseudopodium (see figures 2, 9). Later the connecting pseudopodium breaks, and the new so-called "daughter material," which generally contains a large, variable number of cell-inclusions, migrates independently into the culture medium. The ultimate fate of these bodies, which has been studied *in vivo*, will be discussed in a later portion of this communication.

The nucleus of the main cell at this phase elongates, and still retains its membrane and characteristic differentiation from the rest of the cytoplasm. The mitochondria and other cell-inclusions become aggregated in a central zone of the protoplasm; and dense aggregations of cytomicrosomes around the outer-surface of the nuclear membrane, which are characteristic of the healthy cell, increase in mass (see figure 2).

As a further response to the exhaustion of the medium intense pseudopodial outgrowth becomes characteristic after 65 hours' incubation *in vitro*. This phenomenon is apparently confined only to one pole of the degenerating cell (see figure 3). The pseudopodial movement during this phase of cytolysis is so active that the degenerating cells present an extremely bizarre morphology (see figures 3, 4).

This effect is combined with other profound changes which occur within the interior of the cells. A more detailed observation of the cells at this stage will show that abortive attempts at cell division frequently occur in the form of incomplete amitosis. This phenomenon results in large portions of the cell protoplasm becoming divided, without retaining any nuclear substance (see figure 4). The nucleolus at this stage is occasionally observed to undergo a simple binary fission, generally resulting in the formation of two spherical bodies, which take up their position at the opposite poles of the nucleus.

After three to four days *in vitro*, large accumulations of lipoidal material within the protoplasm are characteristic and pronounced. The finest filaments of the pseudopodia become swollen with fat droplets, while within the main body of the cell the fat aggregates progressively into larger globules as degeneration proceeds (see figures 3, 4, and 5). The actual fusion of the globules with one another was observed *in vivo* where the surface tension forces within the cell were very characteristically demonstrated (see figure 10).

With the nucleus still retaining its continuity, large areas of lipoidal material, together with mitochondria, aggregated at their boundaries, begin to

occupy the main cytoplasmic areas, and even influence the shape of the nucleus by their close proximity (see figures 5 and 6).

At first these areas press against the outer surface of the nuclear membrane, leaving the protoplasm of the pseudopodia relatively free (see figures 5 and 6), but after five to six days' growth *in vitro* these inter-protoplasmic spaces become occupied by the rapid accumulation of fat (see figures 7 and 8).

Eventually cytolysis proceeds so far that all cell-contours begin to disappear, partly by the fusion of the interlacing pseudopodia of adjacent cells, and also by the influence of fat globules, which, by their relative rigidity, resist any alteration in shape by the weak, disintegrating cell membranes (see figure 8). It is interesting to observe that up to this advanced stage of cellular degeneration, that occurs after seven to eight days *in vitro*, and bearing in mind the profound changes in activity and composition of the general protoplasm, the nucleus remains relatively unaltered.

Constrictions were seen to occur in the earlier stages of cytolysis, owing to abnormal amitosis or active pseudopodial movements, and frequently to the close proximity of the cell-inclusions. Many were observed to alter the nuclear contour, but despite these intracellular changes, the nuclear membrane remained intact until the cytoplasm lost all its distinguishing characters.

Chromatolysis of the nucleus was observed to occur after seven to nine days' growth, when the cell-membranes disintegrate and the protoplasm becomes a sponge-like mass of fat globules and vacuoles. Before the disintegration of the nuclear membrane, the chromatin is absorbed, leaving behind only finely staining, reticular vestiges. Combined with the absorption of the chromatin, the nucleolus, which may be seen undergoing amoeboid movement at this stage *in vivo*, hypertrophies, but still possesses a distinct nucleolar membrane (see figure 7).

This structure appears to be supported in the centre of the degenerate nucleus by the reticular remains of the nucleoplasm. The small central body, so characteristic of the healthy nucleolus, remains unaltered during this process, and is clearly depicted in figure 7.

With these final evidences of cytolysis, which occur in from nine to ten days' incubation, the cell membrane disintegrates and disappears, and the cells, long since atypical in morphology, fuse into a sponge-like network of vacuoles and fat (see figure 9).

Even in these final stages of cytolysis the mitochondria and neutral red granules are still present, and are seen to aggregate densely around the vacuoles, as well as in the peripheral regions of the cells. At various intervals many of these intracellular bodies are discharged into the culture medium. Here and there on the surface of the syncytium-like structure, the fat globules burst like soap bubbles (see figure 8), or remain as clear spaces devoid of stainable contents.

For observations on the cytolysis of undifferentiated tissues small portions of tissue were taken from an embryo of 48 hours' incubation in the region of

the somatic mesoderm. After short periods of incubation *in vitro*, in similar media and under the same conditions, it was apparent that the behaviour of this tissue during cytolysis differed very substantially from that already described for differentiated tissues. The cells retain their morphology more readily, giving few examples of extreme distortion in shape.

Examination after three to four days' incubation in an unchanged medium showed that extensive pseudopodial outgrowths were not a characteristic feature, but here and there fine, filiform pseudopodia were occasionally detected. The nucleus almost invariably contained two nucleoli, which were clearly differentiated in the early stages of degeneration, and still persisted after five days' incubation. The comparative scarcity of chromatin was also a character of the nucleus. Abortive attempts at cell division and amitosis, such as frequently occur in differentiated tissues after similar growth *in vitro*, were not observed.

After five days' growth *in vitro* the accumulation of fat was detected. The deposition of fat proceeded by a process entirely different from that in the differentiated, embryonic cells. Instead of the accumulation of a large number of droplets in the cell protoplasm being followed by their gradual coalescence into large fat globules, very large vacuoles formed in the undifferentiated cells into which the fat was gradually liberated (see figure 12). Many cells at this stage of degeneration were observed to contain one of these vacuoles, or rarely more than two. In most instances the nucleus, because of its close proximity to the vacuoles, became distorted and crescent-shaped in contour (see figure 12).

From preparations of this tissue, undifferentiated at the time of explantation, grown *in vitro* under the same conditions as the differentiated embryonic tissue, and incubated for similar extended periods in an unchanged medium, during which degeneration is brought about, several very important observations have been made upon the cytolysis of undifferentiated tissues.

It is at once apparent that these tissues are more resistant to the factors incurring cytolysis, and take a considerably longer period in disintegrating to the degree described in tissues differentiated at the time of explantation. The cytoplasm does not react so profoundly to the chemical changes of the medium, but preserves its contours and homogeneous nature to a remarkable degree. The vacuolated condition observed after four to five days' incubation presents a strong contrast with the differentiated tissues, where the accumulation of fat is a much more rapid process, and may be detected at a much earlier stage in cytolysis.

In order to observe the internal structure and appearance of living unstained cells undergoing advanced cytolysis, isolated cells in the region of the periphery of a seven or eight days' culture of fibroblasts were chosen, and were examined under an oil lens upon a warm stage maintained at body temperature. By means of a direct illumination the protoplasm composing the periphery of a degenerating cell appeared clear, transparent, and homogeneous (see figure 10).

The highly brilliant refractive cytomicrosomes, which occupy the central regions of the cell, were seen undergoing slow, irregular, Brownian-like movements within the deeper cytoplasm of the living cell. The nuclei were observed as dull, greyish, oval bodies when examined by the direct method (see figure 10). Nuclear polymorphism, apparently due to pressure of the superabundant cell-inclusions of the degenerating cell, was a characteristic feature. Occasionally the nucleus of cells in advanced cytolysis was observed to lie in intimate contact with the cell membrane. The nucleus seen in cells at this stage had apparently undergone chromatolysis as no chromatin network, which appears as a dull greyish structure when seen by direct illumination, was visible in the resting nucleus (see figure 10). The nucleolar apparatus, which is imbedded within the viscous nucleoplasm of the living nucleus, appears as a slightly opaque, delicate, swollen-looking structure of irregular contour. Prolonged observations upon the behaviour of this body within the living cell *in vitro* showed that it underwent slow amoeboid movement. The morphology of this nucleolar apparatus in degenerating cells, as seen *in vivo*, compares favourably with that depicted in fixed preparations during similar phases of cytolysis.

The movements of these highly refractive intracellular inclusions of the living degenerating cell are fascinating to observe. As has been previously shown (15) the behaviour and movements of these bodies depends largely upon the temperature at which they are observed. The fusion of larger lipoidal droplets with one another is frequently seen to occur.

When living cultures of three to four days' incubation are subjected to a temperature of 0° C. for over thirty minutes the process of cytolysis is rapidly augmented, particularly with regard to the deposition of fat. Lowering the temperature appears to cause the fat, already deposited in the cytoplasm as small droplets, to coalesce into one or more large globules, which are frequently so enormous that the cell membrane breaks down with their pressure. The nucleus is pushed violently to one side of the cell, where it may lie in intimate contact with the cell wall.

The mitochondria at this stage appear only in a spherical form and are present in greater numbers within the protoplasm forming the periphery of the compact cell. These inclusions are much smaller than the fat droplets and are not so highly refractive. Occasionally several or more mitochondria come to rest and suddenly coalesce with one another, resulting in the formation of a single aggregation. No structure comparable to the Golgi apparatus was detected at this stage.

In order to distinguish between the various types of cytomicrosomes, and to estimate their respective numbers at this stage of degeneration, several isolated fibroblasts were collected and stained *intra vitam* with either neutral red or Janus green. When the former stain was employed the so-called "neutral red" granules displayed great variation in staining reaction. The majority of

these structures coloured up a brilliant red, while others gave a bright yellow. These granules were variable in size and appearance, and were smaller than the refractive lipoidal droplets. The rôle these cell-inclusions play in cellular metabolism is not yet determined.

The mitochondria stained a brilliant green when treated *intra vitam* with Janus green. They showed up as small, spherical bodies, and appeared in fewer numbers than the "neutral red" granules. The recent researches of biochemists and cytologists (13, 14, 25, 32) strongly suggest that these bodies are enzymatic in nature.

Staining reactions with the *intra vitam* dyes showed that as degeneration proceeds the mitochondria gradually decrease in numbers, while the "neutral red" bodies and the lipoidal droplets increase in numbers. Cells in the last stages of cytolysis were found to be practically devoid of mitochondria.

While studying living cells after from four to six days' incubation in an unchanged medium, small, protoplasmic, finger-like processes were sent out from the main body of the cell. The end of these delicate processes proliferated, giving a balloon-like appearance, and many refractive cell-inclusions were, by means of intracellular, protoplasmic currents, swept into the spherical proliferation. Generally after a short interval the thin protoplasmic thread, joining this newly-formed cellular process to the main body of the cell, snapped, and the so-called "daughter portion" migrated independently within the medium, until finally, owing to certain tension forces, this newly-formed body burst, thereby liberating many brilliant, refractive structures, which underwent rapid irregular movements within the culture medium, and ultimately disappeared.

DISCUSSION.

The evidence obtained from this investigation has shown clearly the advantages of the *in vitro* method as a cytological technique, whereby the appearance and behaviour of the degenerating cells may be studied in the isolated condition, either in the fixed or living states.

Detailed observations upon the process of cellular degeneration have resulted in showing very little variation in the behaviour and general appearances of the various cell-types during the same phases of cytolysis, which suggests that the physiological factors incurring this phenomenon were constant; while, on the other hand, the discrepancies already depicted between differentiated and undifferentiated tissues during degeneration may possibly be due to differences in cell-types, combined with certain chemical differences, which we are at the present moment unable to determine.

Nucleolus: One of the most interesting intracellular structures, and one of which very little is known, is that of the nucleolus or plasmasome. Ever since its discovery by Fontala in 1781, this structure has from time to time attracted the attention of cytologists, and consequently a great deal of literature has been

compiled concerning the behaviour and morphology of this apparatus within normal and pathological cells.

In order to attempt to elucidate the apparent functional significance of this intranuclear structure, special attention has been paid to the behaviour of the nucleolus within the healthy and degenerating cell *in vitro*.

The presence of the nucleoli in the cells of undifferentiated tissues of a 48-hour chick embryo *in vitro* is significant, and during a previous investigation (16) it was found that these nuclear structures were a specific morphological character common to the nuclei of 24-hour chick embryos *in vitro*. The appearance of these intranuclear structures is of special interest, as Ludford (22), when observing the undifferentiated cells of the germinal epithelium in *Limnaea stagnalis*, was unable to report the presence of these structures. Apparently the nucleoli were only visible when the germ cells could be definitely recognized as gonocytes.

Our studies *in vitro* have shown clearly that the undifferentiated cell is practically devoid of a chromatin network, so characteristic of the differentiated resting cell-nucleus, while on the other hand the appearance of a binucleolate structure is a specific feature of the undifferentiated tissues.

At the present state of our knowledge concerning the structure of the nucleolus it is difficult to attach any concise physiological significance to this phenomenon, but it is interesting to note that Kunio Sato (34) has also recorded an absence of chromatin within the undifferentiated cells of *Cristaria plicata*, and on this account contends that the nucleolus takes the place of chromatin within the undifferentiated cell, and is also capable of being transformed into chromatin within the nucleus.

The origin of the nucleolus appears to be obscure. Whether this nucleolar apparatus arises *de novo* or from a pre-existing body, which undergoes independent, binary fission during the life of the cell, is not yet determined.

An extra-nuclear origin of the nucleolus was first suggested by Buchner, but later Jorgensen (18) threw doubt upon such a contention. In view of Buchner's work, Gardner (6) recently suggested that the structure, which represents the nucleolus in the auxocyte of *Limulus polyphemus*, is derived from the cytosome. She further maintains that such substances, which have a specific staining reaction, and pass from the cytosome to the nucleus to form the nucleolus, have their origin within the cytoplasm of the auxocyte itself, or may be derived from the tissues surrounding the growing germ cells. Gardner concludes by contending that the nucleolar apparatus is of cytoplasmic origin, and is therefore genetically independent of the nucleus.

While on the other hand several investigators, among whom are Platner (30) and Montgomery (28), contend that the nucleolus or plasmasome is concerned with the production of certain corpuscle-like granules, which may be detected in the cytosome of various cells, recently Hogben (9), while observing the behaviour of this apparatus in Periplaneta, maintains that the nucleolus

from the very beginning of the growth stage extrudes deeply staining particles, which pass through the nuclear membrane into the cytosome.

While critically examining the cytosome of undifferentiated cells of 24- and 28-hour chick embryos, no cytoplasmic granules, which were suggestive of nucleolar substance, could be detected either in the normal or the degenerating cells. The great difficulty lies in determining as to whether these granular bodies are derived from the chromatin network of the nucleus or from the nucleolar structure itself.

As to whether the nucleolar apparatus is a permanent cell body is a matter of controversy. Some authors hold that, when the nuclear membrane is dissolved in the prophase, the nucleolus disintegrates, while, on the other hand, it has been shown that during direct cell division the nucleolar material undergoes profound chemical change, and at the commencement of the prophase it gradually becomes achromatic, but nevertheless persists.

It has also been demonstrated that during amitosis in certain cells the karyosome undergoes independent binary fission at the same time as the nucleus, and this phenomenon has already been seen during the early stages of cytolysis, when amitosis occurs. Whether the nucleolar substance is distributed to each daughter cell after cell-division is apparently a matter of speculation. Until this phenomenon is shown to occur it is impossible to affirm the continuity of this body.

While discussing this problem it is interesting to observe that Carleton (2) has shown that, during mitosis in certain vertebrate cells, although the nucleolus appears to disintegrate, an argentophile core remains intact, and finally passes into each of the newly-formed daughter nuclei, which later develops into the nucleolar apparatus.

The persistent appearance of the karyosome in undifferentiated embryonic tissues *in vitro*, together with its facility to undergo independent binary fission during the life of the cell, strongly suggests the continuity of this intranuclear structure. Up to the present day very little is known concerning the physiology and function of the nucleolus, and it is generally held to be a mere mass of accumulation of waste nuclear material; but judging by its specific appearance and behaviour within the nuclear membrane of normal and pathological tissues it is suggested that it plays an important rôle in the nutrition of the cell.

Observations upon the behaviour of the nucleolus during degeneration show it to be the last structure detected in cells undergoing advanced cytolysis. The characteristic hypertrophy of the nucleolar apparatus, already depicted by us as a special cellular feature during chromatolysis of the atypical degenerating cell, has also been recorded by Saguchi (33), as seen in fixed preparations of pancreatic cells that have degenerated under unknown physiological conditions. The evidence based upon the behaviour of the nucleolus during this phenomenon is very suggestive that it has some functional relationship to the morphology of cells.

While further discussing the functional significance of the nucleolus it is of interest to observe the interpretation of other authors. According to Agar (1) it is commonly held that the nucleoli of the germinal vesicle acts in the capacity of temporary storehouses for chromatin. Agar, however, does not favour any such direct relationship between the nucleoli and the chromatin, as he justly pointed out that only a small portion of the nucleolar substance present in the germinal vesicle could possibly contribute to chromosome formation. Macklin (24) holds that the function of this body is to excite direct nuclear division; and other authors have gone so far as to suggest some direct relationship between the nucleoli and nuclear invaginations, while others have contended that this structure may represent elaborated enzymes.

Centrosphere and Orientation of the Cell-granules: The special orientation of certain cytoplasmic inclusions, so frequently met with in both healthy and pathological, protozoan and metazoan cells, has frequently attracted the attention of cytologists, with the result that many conflicting interpretations have been placed upon this interesting phenomenon.

W. H. Lewis (21) contends that the microcentrum or centrosome plays an active rôle in the arrangement and distribution of various granules within the cell-interior. This author, while observing the appearances and behaviour of degenerating mesenchyme cells *in vitro*, draws particular attention to the arrangement of cell-granules about the centriole and centrosphere of these cells. He further depicts a radial orientation around the centriole structure in fibroblasts undergoing cytolysis *in vitro*. This author, throughout these investigations, shows aggregations of cell-granules around the centrosphere, and not around the nucleus, and on these grounds suggests that the centriole, and not the nucleus, is the centre of cellular metabolic activity.

A swelling or hypertrophy of the centriole apparatus during cytolysis is further described by this investigator.

The hypothesis put forward by Boveri and Van Beneden that the central bodies are of universal occurrence has not been confirmed by recent researches. The microcentrum, which is often seen during the vegetative or resting condition of the healthy cell, could not be depicted in degenerating cells during this period, or even in cells that had entered mitosis.

Detailed observations, however, upon the healthy normal mesenchyme cells of the chick *in vitro* showed structures comparable to the centrosphere lying in the naked cytosome in close proximity to the nucleus; but the intracellular orientation of the mitochondria and other cell granules differs from that depicted by Lewis. An examination of the microphotograph (see figure 13) clearly shows a distribution and orientation of cytoplasmic granules around the outer surface of the nuclear membrane, as well as around the periphery of the centrosphere.

The contention that the cell-mitochondria orientate themselves around the centrosphere, and not the nucleus, is difficult to understand. As we have already seen, doubt has been thrown upon the existence of this intracellular structure

as a permanent cell-organ, entirely absent in the cells of higher plants, and also in certain animal tissues. This apparatus is clearly seen in some young mesenchyme cells, fixed after various periods of growth *in vitro* during inter-mitosis. but detailed examination of our cultures showed that it was not a specific feature pertaining to all cells conforming to this type. Nor can any structure having the appearance of a microcentrum be detected within the protoplasm of the living cell as seen by a direct illumination.

Dense aggregations of mitochondria, such as are seen in many cells, around the outer-surface of the nuclear membrane, have been observed by several authors, and one of us has pointed out in a previous communication (12) that the accumulation of mitochondria at the nuclear-cytoplasmic interface may be a surface tension effect due to the phosphatidal nature of these bodies.

Supposed Origin of Fat: Our observations *in vitro* have shown that lipoidal formation is a predominant feature of the degenerating cell, and this contention concurs with that of other investigators. The presence of fat is such a constant characteristic peculiar to cells undergoing cytolysis that these lipoidal globules are frequently termed by authors "degenerative granules." Lambert and Hanes (19), however, also observe a marked increase in the numbers of these bodies with the age of the cell *in vitro*, and do not regard the presence of these cytoplasmic components as evidence of degeneration, as is generally supposed, but look upon their presence as indicative of active, anabolic function upon the part of the cells. They further contend that these fat grains are synthesized by the cell from the soluble elements of neutral fat present in the plasma itself.

Few pathologists, among whom are Ellis (5), suggest that the lipoid, which occurs in fatty degeneration, is carried to the cells by the medium of the blood. In view of these contentions, it is interesting to observe that Ignatowitch (17) has demonstrated that plasma plays no rôle in resynthesizing fat, as this substance accumulates in cells when cultivated in inorganic media, and therefore one must admit the possibility of an intracellular origin of fat from the protoplasm itself.

As tissues growing *in vitro* in organic media are completely cut off from vascular control, it is difficult to contend with the suggestion of Ellis. The intracellular origin of fat was put forward by Lambert and Hanes (20), and later supported by Scott (35), who contended that the cell mitochondria may, under certain pathological conditions, transform into droplets with pronounced fatty and lipoidal properties.

Recently, while studying the behaviour and structure of mitochondria within living cells *in vitro,* the rod-shaped mitochondria were observed to break down into small spherical bodies, which coalesced with one another, and eventually gave rise to a large globule, which stained brilliantly with Sudan 3.

Vacuole Formation. Observations during this investigation have demonstrated that another dominant feature of cellular degeneration, besides that of fat formation, is that of vacuolation.

According to Metcalfe (27) the origin of vacuoles appears to be obscure,

but this author puts forward the suggestion that new vacuoles are formed from the fusion of several smaller ones, and Mast (26) supports him in this contention.

Day (4), while studying vacuole formation in certain Protozoa, states that these structures owe their origin to the coalescence of droplets of soluble katabolic waste.

Our own investigations, based upon *in vitro* study of this phenomenon, indicate that new vacuoles arise *de novo,* and are formed within the cell-protoplasm at random. We have further observed that the hydrogen ion concentration of the culture medium plays an important rôle in vacuole formation.

It is found that fibroblasts and other cell-types, that have been cultivated for 48 hours in a medium slightly acid, give extensive vacuolation without any evidence of cytolysis, such as fat accumulation, and the characteristic signs of chromatolysis (see figure 14). In a medium of an hydrogen ion concentration of 5·5 to 6·5 this vacuolation is very pronounced, even after only 20 hours' incubation *in vitro.* In undifferentiated cells one or two large vacuoles are generally present, but in tissues which have differentiated a polyvacuolated condition is more common.

Nuclear Phenomena: Gaule (8), Nussbaum (29), and Platner (30), towards the close of the last century, were among the earliest investigators to describe nuclear changes occurring in cells undergoing physiological degeneration. Since then other authors have briefly touched upon certain differences in the appearance and structure of the nucleus incurred by that process, until recently, when Saguchi (33) observed this process in great detail in degenerating pancreatic cells.

The evidence obtained by these workers concerning intranuclear behaviour during cytolysis is somewhat conflicting. The earlier authors, when working upon pancreatic cells and other tissues, describe chromatolysis as being a characteristic feature accompanying degeneration, while Saguchi, records the first changes occurring as hyperchromasy, followed by chromatic separation. He further concludes that this process is characterized by a flowing together of the chromatin corpuscles, which leads to the formation of large chromatin granules, which anastomose, and giving a dense chromatin network around the inner surface of the nuclear membrane.

Our studies upon degenerating cells *in vitro* have shown that chromatolysis is a specific feature accompanying all cells undergoing advanced cytolysis, and in this respect we are therefore in accord with the observations of the earlier workers. Hyperchromasy of the nuclear wall is not depicted during this process in either differentiated or undifferentiated tissues. The small chromatin corpuscles, which were formed in consequence of chromatolysis in the degenerating nucleus, gradually refuse to take up the stain, and become totally achromatic before the occurrence of cell-death.

The phenomenon of karyolysis, first described by Macallum (23), and stated by some authors to be a feature common to the degenerating nucleus, was not met with during this investigation.

Garnier (7) was among the first investigators to observe that amitosis accompanies degenerative changes in the cell, and most authors are in accord that this phenomenon is generally met with in pathological conditions, except Macklin (24), who regards nuclear amitosis as merely a change in form of the healthy cell.

Holmes (10) recently noticed amitosis with cytoplasmic division in the ectoderm cells of frogs that had been cultivated for prolonged periods *in vitro*, in an unchanged medium, and also considers this phenomenon as indicating a declining activity.

Our own observations *in vitro* showed that the phenomenon of amitosis is frequent in cells undergoing cytolysis, and, moreover, that it occurs especially at a stage when tension forces are set up in the cell-interior by the increase in volume of the hypertrophied cell-inclusions. Consequently one may suggest that nuclear amitosis, followed by equal or unequal division of the cytoplasm, may represent an endeavour to neutralize these new distorting forces within the degenerating cell.

Another interesting feature met with in cells undergoing advanced cytolysis *in vitro* is that of nuclear distortion. Champy and Carleton (3) contend that the shape of the nucleus is a surface tension effect, and assume that, when surface tension is equal over the whole area of the nuclear membrane, the nucleus tends to assume a spherical shape; and moreover that, when the surface tension varies over the interface between nucleus and cytoplasm, nuclear polymorphism may develop. This phenomenon of nuclear distortion, such as is described by these authors as occurring in healthy cells, was not apparently brought about in our cultures, either of normal or degenerating cells, by surface tension forces. The deformation met with in pathological conditions was apparently incurred by the pressure of the accumulated cytoplasmic inclusions against the nuclear membrane.

Another interesting process, occasionally depicted in degenerating cells, while not seen in normal types, is that of contact between the nucleus and the cell membrane. In view of this effect it is interesting to note the recent contention of Champy and Carleton (3), who have brought forward evidence which suggests that a mutual repulsion exists in the healthy cell between the nucleus and the plasma membrane.

SUMMARY.

1. Detailed, comparative, cytological observations have been made upon the phenomenon of cytolysis in cells that were either differentiated or undifferentiated at the time of explanation *in vitro*.

2. The behaviour and appearance of undifferentiated cells during degeneration have been found to differ from that of differentiated cells. The former show a remarkable resistance to cytolysis when incubated for similar prolonged periods and under the same conditions as differentiated tissues.

3. The process of lipoidal formation, vacuolation, chromatolysis, and atypical nuclear phenomena have been described in detail.

4. The behaviour and functional significance of the nucleolus within the normal and degenerating cell has been discussed.

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EXPLANATION OF PLATES 1–5.

All microphotographs (except those of Figure 1) depict fibroblasts from the heart of a seven days' old chick embryo that have been fixed and stained by the Heidenhain process after undergoing cellular degeneration for certain periods in an unchanged medium of fowl plasma and chicken embryonic extract.

Figure 1. Microphotograph of normal fibroblast, taken around the periphery of a healthy culture after 48 hours' growth *in vitro*. Observe structure of healthy cell.

Figure 2. The same after 75 hours' incubation *in vitro*. Note atypical cell outline, as well as the aggregation of cytomicrosomes within the centre of cell, and accumulations around the nucleus. Observe needle-like pseudopodia. Note appearance of anterior cell and observe curious unequal division of protoplasm.

Figures 3 and 4. The same depicting further advanced stages of cytolysis demonstrating active pseudopodial outgrowths, from one pole of cell only. Note the increased number of fat globules and vacuole structures. In Figure 3 the degenerating cell may be seen undergoing amitosis; and in Figure 4 observe an equal division of cytoplasm without nuclear material.

Figures 5 and 6. The same after four days' growth *in vitro*. The formation of large lipoidal globules, together with atypical morphology of cells, is characteristic of this phase. Observe persistence of nuclear membrane, and also distortion of nucleus apparently due to pressure from the hypertrophied cell-inclusions. Spherical and rod-like mitochondria may be detected within the protoplasm.

Figures 7 and 8. Microphotographs demonstrating same. Last stages of degeneration after eight days' incubation in unchanged medium. Note increased fatty formation, which has affected the general contour of cells. In Figure 7 chromatolysis of the nucleus is clearly seen, but, nevertheless, the nuclear membrane still remains intact. The nucleolar apparatus has hypertrophied, but the central structure of the nucleolus is clearly depicted. Small cell-inclusions are seen in the protoplasm within the peripheral region of the cell. In Figure 8 a large fat globule may be seen discharging into the medium.

Figure 9. Microphotograph showing unequal budding of the cytoplasm after 75 hours' incubation *in vitro*. The same phenomenon is depicted here as already described in Figure 2.

EXPLANATION OF PLATE 6.

Photograph showing lipoidal formation in living unstained and fixed degenerating fibroblasts that have been cultivated *in vitro*, in an unchanged medium of plasma and embryonic extract.

Figure 10. Showing photograph of unstained, living fibroblast. Large fat droplets may be detected within the deeper portions of the clear, homogeneous protoplasm. Small granules, either mitochondria or "Neutral Red" grains, show up as highly refractive bodies in the peripheral regions of the cell, after incubation for 5 days.

Figure 11. Depicting enormous fat globules in degenerated fibroblasts after exposure to a temperature of 0° C. for a period of half an hour.

EXPLANATION OF PLATE 7.

Microphotograph of fixed and stained cells obtained from region of somatic mesoderm of 48-hour chick embryo. Cultivated *in vitro* in plasma and embryonic extract.

Figure 12. Depicting cells, undifferentiated at the time of implantation, fixed after five days' growth *in vitro* in an unchanged medium. Note curious single vacuole formation, observe clearly differentiated nucleus, together with absence of chromatin. Observe the two well-defined nucleolar structures common to each individual cell. Also see occasional formation of fat within vacuoles.

EXPLANATION OF PLATE 8.

Depicting microphotographs of differentiated chick embryonic cells fixed after cultivation *in vitro*. The background in Figures 13 and 14 has been blocked out.

Figure 13. Young mesenchyme cell. Note orientation of cell granules around nuclear membrane and centrosphere.

Figure 14. Depicting histiocyte (anterior), together with a young fibroblast. Observe dense vacuole formation, apparently due to acidity of the medium.

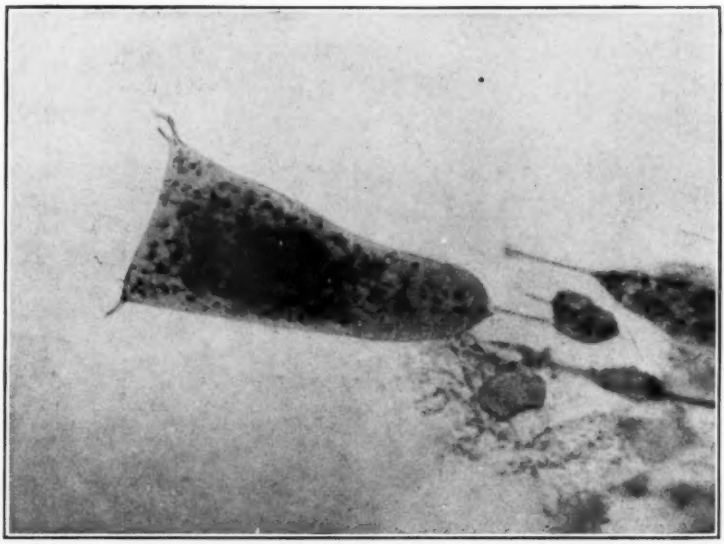

Plate 1. Fig. 1.

Plate 1. Fig. 2.

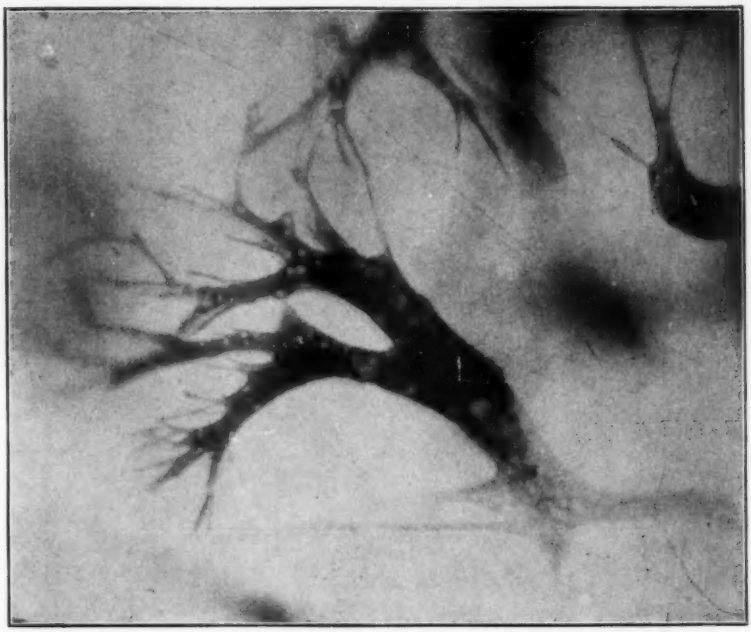

Plate 2. Fig. 3.

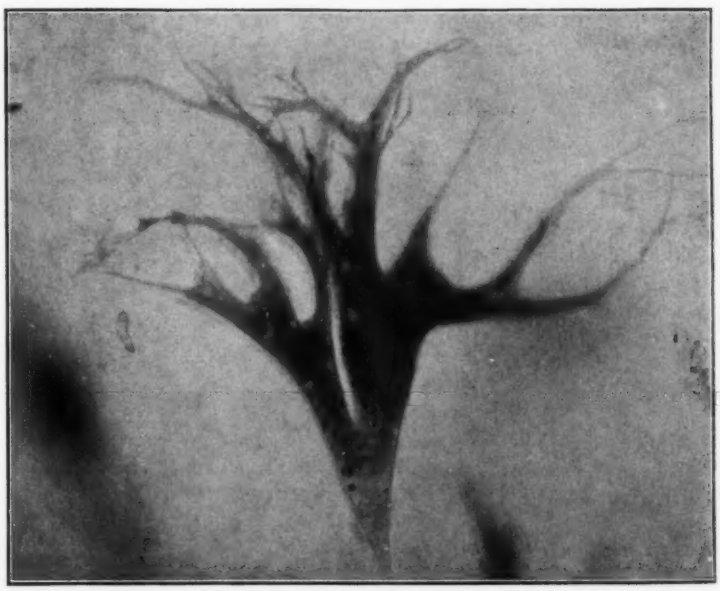

Plate 2. Fig. 4.

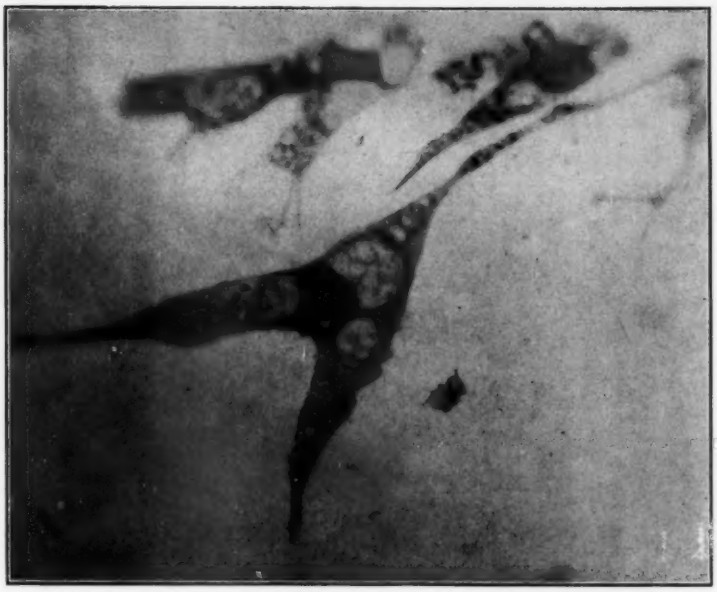

Plate 3. Fig. 5.

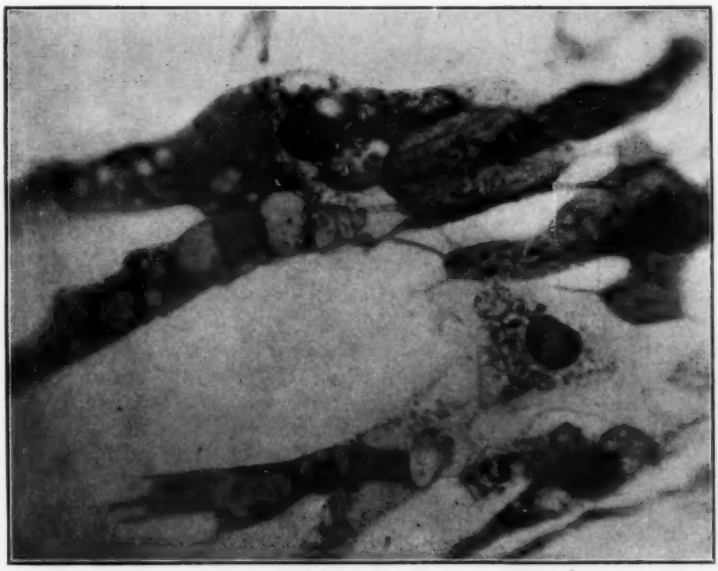

Plate 3. Fig. 6.

Plate 4. Fig. 7.

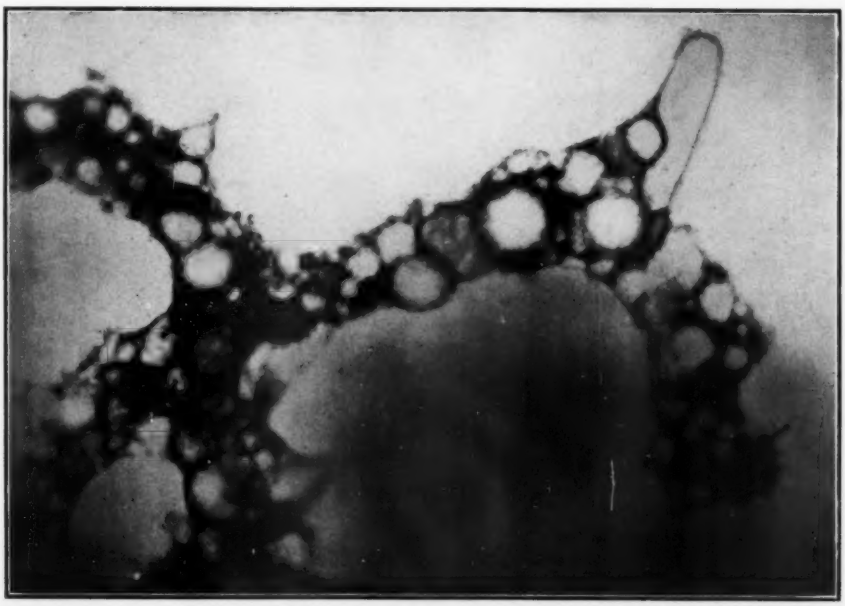

Plate 4. Fig. 8.

Plate 5. Fig. 9.

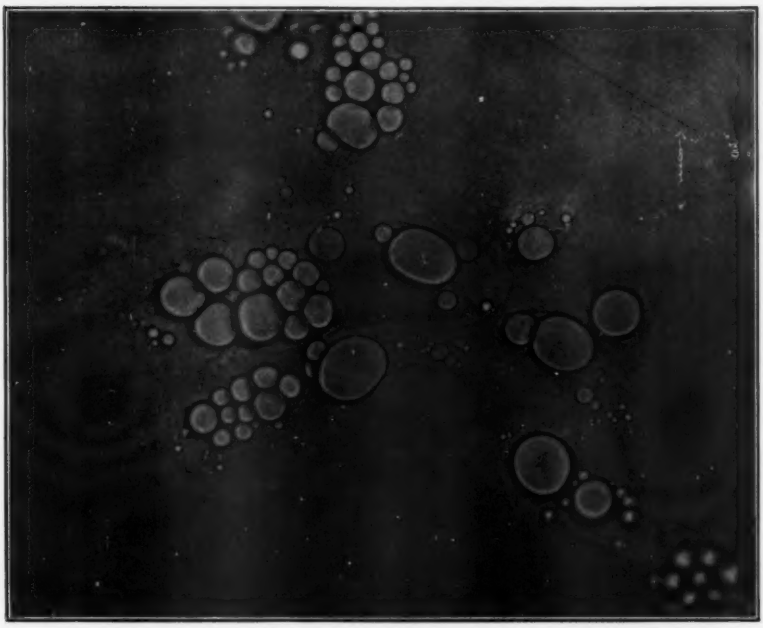

Plate 6. Fig. 10.

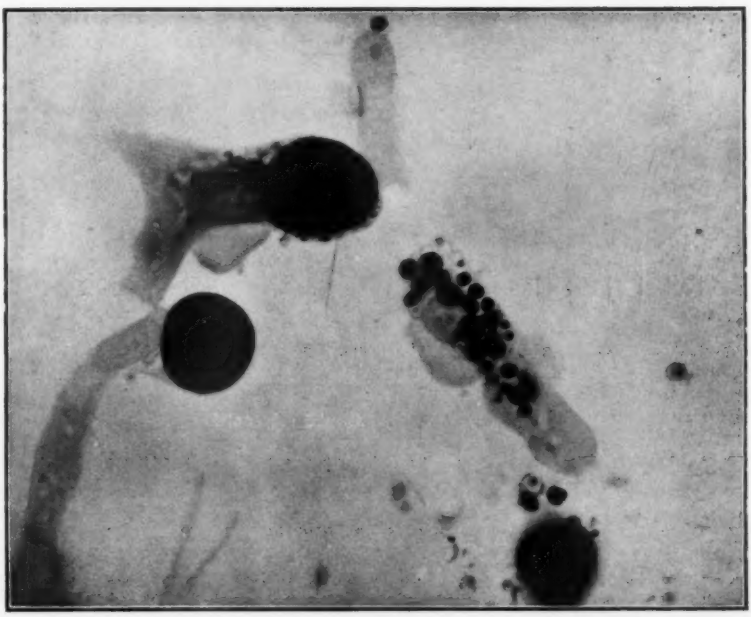

Plate 6. Fig. 11.

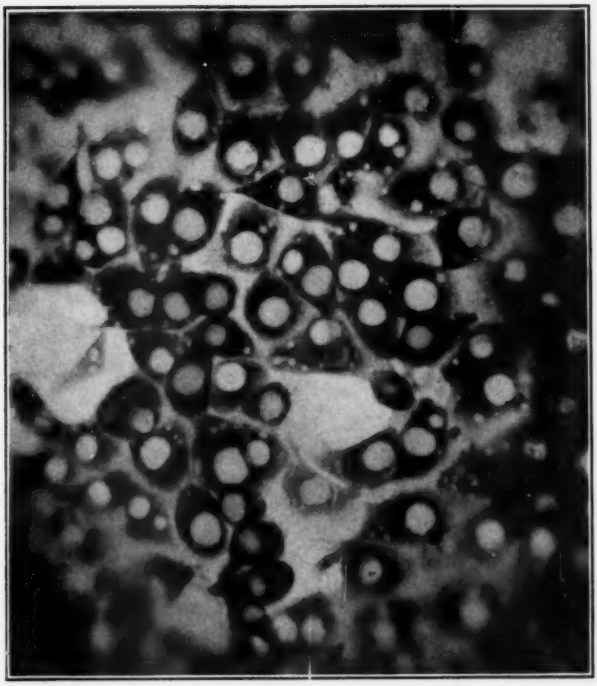

Plate 7. Fig. 12.

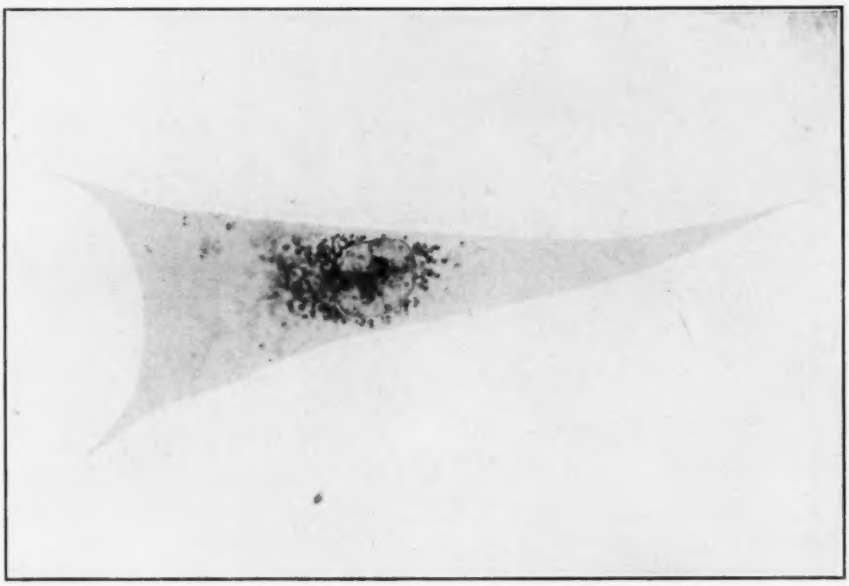

Plate 8. Fig. 13.

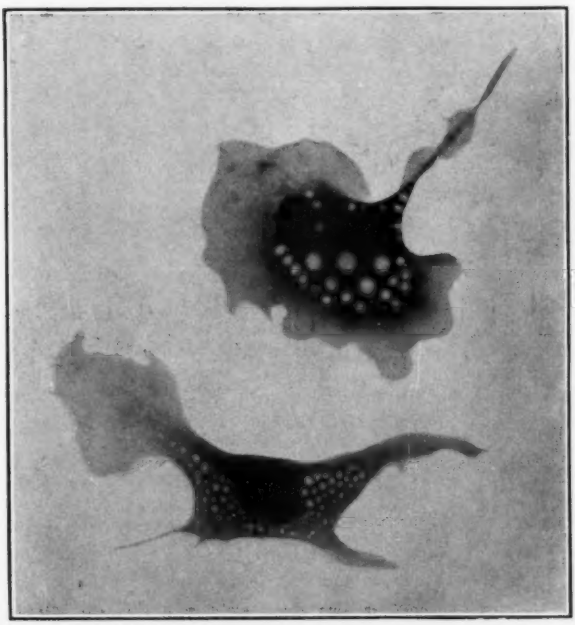

Plate 8. Fig. 14.

THE FRACTIONATION OF AUSTRALIAN SNAKE VENOMS[1]

I. THE VENOM OF THE DEATH ADDER *(ACANTHOPHIS ANTARCTICUS).*

by

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The venoms of poisonous snakes are extremely complex, both in composition and in respect of their toxic activities. Venom must be regarded as a highly specialized type of saliva, whose physiological functions from the ophidian standpoint are the rapid immobilization of prey and (according to Delezenne, 1902) its preparation for the further action of the digestive juices. The venoms of various snakes have been shown to contain water, salts (chiefly chlorides and phosphates), pigment, proteins coagulable by heat, and proteoses. Generally speaking, the toxic properties of venom have been thought to be associated with the protein and the proteoses of the venoms, though Faust (1907) claims that he has separated from cobra venom a nitrogen-free substance which resembles a glucoside in composition, and which is highly toxic.

The toxic activities of snake venoms are no less complex. Many possess powerful haemagglutinic, haemolytic, and cytolytic activities; some when injected intravenously cause coagulation in the vessels, and others delay or prevent the normal coagulation of shed blood; most venoms cause some degree of haemorrhage, possibly related to lytic action on the endothelial lining of the vessels; nearly all have some neurotoxic action, either central or peripheral; some contain proteolytic or lipolytic ferments; and, finally, some reduce the resistance of the tissues and permit of the growth of micro-organisms at and around the site of injection.

In the venoms of the Australian elapines, which are the subject of the present studies, coagulant or anticoagulant, haemolytic, neurotoxic, and haemorrhagic activities are present in nearly all. Martin and McGarvie Smith (1892), working with the venom of the Australian black snake *(Pseudechis porphyriacus)*, found that the neurotoxic activity of this venom appeared to reside in the proteose fraction, which they separated by removing the coagulable protein by heating to 90°C., or by allowing venom to remain under a large

[1] This research was carried out under a grant from the Department of Health of the Commonwealth of Australia.

volume of absolute alcohol for some weeks, and subsequently extracting it with 5% NaCl solution. Later, Martin (1896[2]) separated the coagulable proteins by ultrafiltration at high pressure, and showed that the filtrate, which contained no coagulable protein, possessed the neurotoxic activity of the venom, but did not cause coagulation when injected intravenously. The haemorrhagic and haemotoxic effects which followed the injection of the whole venom in dogs were not produced by this fraction of the venom.

The methods available for quantitative fractionation of venoms are somewhat limited. Methods involving the use of heat are, as will be seen, unsatisfactory, since they involve grave loss of toxicity. The removal of haemolysins or cytolysins by absorption with appropriate cells (Flexner and Noguchi, 1905) is unsuitable, since it involves the addition of unknown quantities of protein substances. Isolation of neurotoxin by removing the haemolysin in combination with lecithin as a "lecithide" (Kyes, 1903) does not appear to be applicable as a quantitative method. Dialysis or ultrafiltration at moderate pressures, 300–450 mm. Hg., through pyroxylin membranes made by Adair's method (1915), does not give quantitative separation of the coagulable protein from the proteoses of venom, since with membranes sufficiently permeable to permit of the passage of proteoses, protein coagulable by heat also passes through. We have not so far attempted ultrafiltration at high pressures (Martin, 1896[1]), but with this method adsorption to the filter candle must necessarily involve much loss. Precipitation with salts of the heavy metals and with dialyzed iron have not, in our experience, yielded satisfactory results, since much proteose appears to be adsorbed or precipitated, and, despite many washings, cannot be removed from the precipitate.

In our preliminary experiments we used precipitation by heat, and subsequent fractionation of the filtrate by half and full saturation with ammonium sulphate. This method was found to have two disadvantages. There was definite loss of activity, caused by heating the venom, and the alteration of the coagulable proteins by heat made its uncertain after re-solution with dilute alkali, whether their toxic activity had not been greatly impaired in the process.

The effect of heat on the toxicity of the proteose fraction of death adder venom was tested by subcutaneous injections in guinea-pigs and by intravenous injections in mice.

Titration of the Proteose Fraction of Heated Death Adder Venom.

A 1% solution of venom was made in water, and equal volumes of this were measured into five tubes. One of these was kept as a control, and the others were heated for 5, 10, 20, and 30 minutes respectively in a boiling water bath. The actual temperature attained by the venom solution was 94–95°C., being somewhat higher in the tubes which were heated longer. The precipitate of coagulated protein was centrifuged down, and the clear solution pipetted off. Two

washings of the precipitate were added to the filtrates, which were then made up to the requisite volume. The filtrates were injected subcutaneously in the abdominal walls of guinea-pigs, weighing from 250 to 300 grammes. The dose was arranged in multiples of the certainly lethal dose for guinea-pigs of this size (0·015 mgm. per 100 grammes of body weight).

The filtrate from venom heated for five minutes did not kill regularly in amounts equivalent to 2 C.L.D.'s, though doses equivalent to $1\frac{1}{4}$ and $1\frac{1}{2}$ C.L.D. sometimes caused death, and almost invariably produced symptoms. The filtrates from venom heated from 10, 20, and 30 minutes did not produce any symptoms in doses equivalent to $2\frac{1}{2}$, 3, and 4 C.L.D.'s.

Injected intravenously into the tail veins of mice weighing from 15 to 30 grammes, in multiples of the C.L.D. (0·008 per 20 grammes), the results set out in Table 1 were obtained.

TABLE 1.

Results of the Intravenous Injection of Filtrates from Heated Death Adder Venom in Mice.

Times for which venom was heated.	Number of animals injected.	Dose in equivalents of C.L.D.s.	Result.
5 minutes	7	2	All dead in $5\frac{1}{2}$ hours.
	4	1·25	Two died between the first and fourth day and two survived.
10 minutes	7	4	All died in from 40 minutes to 6 hours.
	6	3	All died in from $1\frac{1}{2}$ to 6 hours.
	8	2	Five died before the fourth day and three survived.
20 minutes	7	6	All died in from 7 hours to the sixth day.
	4	4	One died on the first day and three survived.
30 minutes	7	8	All died between 2 and 20 hours.
	7	6	Five died between 5 hours and the fourth day.

The certainly lethal doses of death adder venom for mice and guinea-pigs were estimated by injecting groups of animals, 20 or more in number, with fairly closely spaced doses near the lethal dose (Kellaway, 1929[1]). The dose taken as the C.L.D. is that which kills 100% of animals. Though the dosage of proteose fraction used in these experiments was calculated on the assumption that all the toxicity of the venom resided in this fraction, the loss observed was out of all proportion to the loss in the precipitate, which is roughly one-fifth of the weight of the venom. Apart, therefore, from its effect on the toxicity of the coagulable proteins of the venom, heating greatly lowers the toxicity of the proteose fraction, and makes any method involving its use undesirable as far as this venom is concerned.

Tests of the haemolytic activity of the filtrates were also carried out, using washed guinea-pig red blood corpuscles. It was found that, whereas 5 and 10

minute filtrates were only slightly less active than the unheated venom, there was considerable loss of activity in the filtrates from venom heated for 20 and 30 minutes. Similar findings were obtained in tests on the stimulant action of the filtrates on the isolated uterus of the guinea-pig.

The method which we have adopted for the quantitative studies recorded in this paper consists in the extraction of dry powdered venom with a suitable mixture of ethyl alcohol and water. By carrying out the extraction at 0°C. the coagulable protein which is precipitated is completely soluble in water. The percentage of alcohol and water to be used for fractionation is determined by trial, that mixture being used which gives a precipitate of insoluble matter of the same order as the amount of protein coagulated by heat.

The Percentage of Coagulable Protein in the Venom of the Death Adder.

A 1% solution of venom was made in distilled water, measured by pipette into small tubes weighed on the micro-balance, and heated in a boiling water bath for 45 minutes. The precipitate was centrifuged down in the same tubes, washed thoroughly with water and alcohol, and dried in an oven at 100–105°C. After cooling the tubes were re-weighed. The percentage of coagulable protein was determined in three different samples of dry, powdered venom, with the following results:

	(1)	(2)	(3)
	17·31	19·9	18·83
	17·27	20·4	18·32
	17·59		
	17·84		
mean	17·5	20·2	18·6

The amount of venom used for each single micro-determination was only 4–5 mgms., but it is possible that part of the variation observed in different samples was due to genuine variation in the composition of the venom.

The Percentage of Venom Precipitated by Various Strengths of Alcohol.

The procedure adopted was as follows: Alcohol of the necessary strength was accurately made up by volume, and cooled in the refrigerator. Dry powdered venom was weighed in small tubes. The alcohol, in amounts of 2–5 c.cm., was pipetted on to the venom, and the tubes were corked tightly and left overnight in the refrigerator. Next morning the insoluble residue of venom was stirred up and then centrifuged down. The supernatant liquid was pipetted off, and the insoluble fraction was washed six times by centrifugation, using alcohol of the same strength, the washings being added to the original alcohol extract (soluble fraction). The soluble fraction was now taken to dryness in a vacuum desiccator, and the tubes containing the insoluble fraction were treated in the same way. By weighing, the percentage of the insoluble fraction was determined. With varying strengths of alcohol the following results were obtained:

% of Alcohol by Volume.		% by Weight of Insoluble Fraction.					
55%		25·93					
		26·08					
	mean	26·0					
45%	(1)	17·13	(2)	18·23	(3)	18·55	
		18·80		18·85		18·71	
				18·51		18·26	
						19·20	
	mean	17·9		18·5		18·7	
40%		15·46					
		15·63					
	mean	15·5					

The toxicity of all these various fractions was tested by subcutaneous injection, using only a few guinea-pigs for each, and also by their stimulant action on the isolated uterus of virgin guinea-pigs. In many also coagulation and haemolysis tests were carried out on both fractions. The results clearly indicated that satisfactory fractionation had been obtained only with 45% alcohol. With 50% strength the insoluble fraction contained much proteose, and with 40% not all of the coagulable protein.

Estimation of the percentage of protein coagulable by heat in the soluble and insoluble fractions from two separate fractionations with 45% alcohol showed that 86% of the insoluble fractions was coagulable by heat, while in the soluble fractions the percentages were 7·5% and 4·4%. It will be seen, therefore, that the separation of coagulable protein from proteose by this fractionation, though not complete, is sufficiently sharp to allow of deduction in regard to the properties of the fractions.

Titration of the Fractions by Subcutaneous Injection in Guinea-pigs.

The guinea-pigs used varied between 250 and 300 grammes in weight, and for animals of this size Kellaway (1929[1]) has determined the "characteristic" of the venom by subcutaneous injection. With a dose of 0·015 mgm. per 100 grammes, 100% of animals died in less than 3 days, whereas with a dose of 0·012 mgm. 14 out of 22 animals died in less than 5 days and 8 survived. For testing, the insoluble fraction was made up to contain the insoluble part of 1 mgm. of venom per c.cm., and the soluble fraction to contain the soluble part of 0·1 mgm. of venom per c.cm. The injection of groups of 10 animals with soluble fraction in dose equivalent to 0·015 mgm. of the whole venom per 100 grammes (1 C.L.D.), showed that the loss of the insoluble material by the venom had not involved any great loss of toxicity, since in experiment 8, and in two others, all the animals died by the second and third day. With the first of these samples 12 animals which received 1·2 C.L.D. were all dead by the second

day. On the other hand, groups of animals injected with the insoluble fraction were killed regularly in 24 hours when amounts equivalent to 20 C.L.D.'s of the venom were used. Amounts equivalent to 15 C.L.D.'s also killed regularly in 2–3 days, and with 12 and 10 C.L.D.'s only 1 of 6 animals died in each group.

The symptoms and post-mortem findings were closely similar in animals killed with either the soluble or insoluble fractions, and neither showed any gross differences from those in animals killed by subcutaneous injection of the whole venom. The most striking features were the respiratory symptoms associated with massive haemorrhages in the lungs and the congested adrenals.

The "haemorrhagin" in the venom is not segregated in one or other fraction, but is distributed in both, and the "neurotoxin," which is chiefly responsible for the fatal issue in those animals which died, is mainly present in the soluble fraction. The insoluble fraction when administered subcutaneously is about one-third as active as the whole venom (weight for weight) in its toxicity for guinea-pigs.

When the insoluble fraction was tested by intravenous injection in guinea-pigs, the results obtained were strikingly different. The C.L.D. of the venom by this route is about 0·006 mgm. per 100 grammes, and insoluble fraction equivalent to 4 C.L.D. killed 2 of 12 animals on the third and fourth days, and caused severe symptoms in several of the survivors. Doses equivalent to 6 C.L.D. regularly caused death in 2 days.

Titration of Fractions by Intravenous Injection in Mice.

The C.L.D. of the venom for mice of from 15 to 25 grammes is 0·008 mgm. per 20 grammes. A dose of 0·006 mgm. kills 70%, one of 0·005 mgm. 50%, and one of 0·004 mgm. per 20 grammes 40% of the animals injected intravenously (Kellaway, 1929[1]).

The insoluble fraction injected intravenously in dose equivalent to 10 C.L.D.'s kills regularly in less than 5 hours, and in doses equivalent to 5 C.L.D.'s in less than 20 hours. Animals injected with insoluble fraction equivalent to 2 C.L.D.'s survived without symptoms. The soluble fraction in a dose equivalent to 1 C.L.D. killed 8 mice, all but 3, within 4 hours.

By subcutaneous injection a dose of the same sample of the insoluble fraction equivalent to 20 C.L.D.'s (1 C.L.D. = 0·014 mgm. per 20 grammes), killed 4 of 6 animals, while 4 mice injected with the equivalent of 15 C.L.D.'s survived without symptoms.

As in the guinea-pig experiments, there is a striking difference between intravenous and subcutaneous injection. By intravenous injection the insoluble fraction has a toxicity equal to or grater than that of the whole venom (weight for weight), while by subcutaneous injection it has only about one-quarter of the toxicity of the whole venom. For the mouse the proportion between the fatal dose of the whole venom by intravenous and subcutaneous injection is

1 : 1·75, and it seems that this difference in toxicity may be due to the non-absorption or slow absorption of the less diffusible coagulable protein of the venom when this is administered subcutaneously, its toxic effect coming into play when the venom is injected intravenously. It should be recognized that death in this latter case is not due to intravascular coagulation, the venom even in very large doses having no coagulant action *in vivo* (Kellaway, 1929[2]).

Titration of the Fractions by Haemolysis.

We used for these tests a 3% suspension in normal saline of four times washed guinea-pig red blood corpuscles. The volume of the haemolytic system was 0·3 c.cm. (0·1 of saline, 0·1 of saline containing venom or fractions of venom, and 0·1 c.cm. of red blood corpuscles). The tests were carried out in an incubator at 37°C., the tubes being observed every half-hour. The venom was tested in amounts of 0·1, 0·01, 0·001, 0·0001, and 0·00001 mgm., and the fractions were made up so as to be equivalent to these amounts of the whole venom. For example, the soluble fraction equivalent to 0·1 mgm. contained in 0·1 c.cm. about four-fifths of this weight of dry soluble fraction, and the equivalent solution of insoluble fraction contained about one-fifth of a milligramme of dry insoluble fraction.

The variations observed in the times necessary for complete haemolysis are indicated in Table 2.

TABLE 2.

Times at which Haemolysis was Observed to be Complete in Tests with Death Adder Venom and Fractions of this Venom.

Amount of venom in mgm.	Haemolysis, times in hours. Soluble fraction.	Insoluble fraction.	Whole venom.
0·1	1 to 2	1 to 2½	1 to 2
0·01	2 to 3½	2 to 4	1½ to 4
0·001	3 to 5	2½ to between 5 and 20	2½ to 4½
0·0001	no haemolysis	no haemolysis	between 5 and 20
0·00001	no haemolysis	no haemolysis	no haemolysis

There is here no evidence of segregation of the haemolytic activity of the venom in either fraction, making due allowance for the fact that the weights of dry insoluble fraction tested are one-fifth of those of the whole venom and one-quarter those of the soluble fraction. The observed haemolysis accords with the view that haemolytic activity is evenly distributed between the two fractions.

Titration of the Fractions by Tests of Coagulant and Anticoagulant Activity.

A number of experiments were carried out by Lamb's (1903) method, using citrated plasmas of the sheep and guinea-pig. These were prepared by shedding blood into an equal volume of 2% sodium citrate solution in normal saline, and spinning down the corpuscles. Various amounts of venom (0·1, 0·01, 0·001, 0·0001, and 0·00001 mgm.) in 0·1 c.cm. of saline and equivalent amounts of

the two fractions, were placed in a series of tubes 7·5 c.cm. long and 12 mm. internal diameter, which were specially cleaned by boiling in aqua regia and subsequent washing in many changes of distilled water. To each was added 0·2 c.cm. of plasma. After incubation for 1 hour at 37°C., that amount of 0·5 or 1% $CaCl_2$ was added, which produced rapid clotting in control tubes, and the clotting times in the tubes were observed at room temperature, 20–21°C. The results of a number of tests of different fractionations were uniform, and in Table 3 is set out a typical result with guinea-pig plasma. The clotting time of control tubes, to which 0·2 c.cm. of 1% solution of $CaCl_2$ had been added, was 4 minutes.

TABLE 3.

Coagulation Times of Plasma with Death Adder Venom and its Fractions.

Amount of venom in mgms.	Coagulation times in minutes. Soluble fraction. Without $CaCl_2$	Soluble fraction. With $CaCl_2$	Insoluble fraction. Without $CaCl_2$	Insoluble fraction. With $CaCl_2$	Whole venom. Without $CaCl_2$	Whole venom. With $CaCl_2$
0·1	no clotting	8 (soft)	30 (soft)		30 (soft)	
0·01	„	8 (soft)	no clotting	6 (soft)	no clotting	5 (soft)
0·001	„	8	„	5	„	5 (soft)
0·0001	„	4	„	4½	„	5
0·00001	„	4	„	4½	„	4

It has been shown by one of us (Kellaway, 1929[2]) that the venom of the death adder (though even in large doses intravenously it fails to cause intravascular clotting) has a feeble coagulant action *in vitro*, while in smaller amounts it exerts a weak anticoagulant effect. Fractionation appears to have separated these two activities of the venom. The coagulant property is associated with the insoluble fraction, while the soluble fraction has a feeble anticoagulant action.

Titration of the Stimulant Action on the Isolated Plain Muscle of the Guinea-pig.

All the Australian elapine venoms have a stimulant action on plain muscle (Kellaway, 1929[3]). This resembles the anaphylactic contraction of the guinea-pig uterus in its time relations, and in respect of the desensitization which follows the response. The plain muscle having once reacted fails to respond to the same dose of the venom, or to an equivalent stimulant dose of another venom. We have tested the fractions on the isolated uteri of virgin guinea-pigs suspended in a bath containing 50 c.cm. of oxygenated Ringer's solution at 37°C. Advantage has been taken of the approximately equal sensitiveness of the two uterine horns, one being used for testing a fraction and the other to ascertain the sensitiveness of the uterus to death adder venom. The extent of contraction has been estimated by subsequent response of the uterus to histamine.

Titration, using a number of guinea-pigs, showed that the soluble fraction contained most of the stimulant activity of the venom. The soluble fraction from

0·006 mgm. of venom was more active than 0·005 mgm., and less active than 0·007 mgm. of death adder venom. On the other hand, the insoluble fraction also contained some of the stimulant substance. The insoluble fraction from 0·04 mgm. of venom was more active than 0·004 mgm. of death adder venom, and that from 0·02 mgm. of venom less active than 0·003 mgm. of whole venom. Though the error in such assays is considerable, about nine-tenths of the stimulant activity of the venom appears to reside in the soluble fraction.

Titration in Frogs.

The venom of the death adder has a definite peripheral, curare-like action, and the experiment of Claude Bernard (1857) may be repeated in the frog, using this venom.

Experiment 1.

A frog *(Limnodynastes dorsalis)*, weighing 15 grammes, was pithed, and a tight ligature was tied round its right thigh as high as possible. Four mgm. of death adder poison was now injected into the dorsal lymph sac. The left sciatic nerve was exposed and stimulated, using a du Bois Reymond coil with variable break, the rate of interruption being set at 180 per minute. The distances of the secondary from the primary necessary for effective contraction of muscle when the nerve and muscle were stimulated were as follows:

Time after injection of venom.	Distance of primary from secondary. For nerve.	For muscle.
38 minutes	23 cm. (good)	24 cm.
50 minutes	17 cm. (feeble)	22 cm.
1 hour 30 minutes	0 cm. (no contraction)	22 cm.

At 1 hour 30 minutes, when the heart was still beating feebly, but the frog no longer responded to mechanical stimuli, the right sciatic nerve was exposed and stimulated, and the muscles gave a good contraction at 24 centimetres when either the nerve or the muscle was stimulated.

This experiment was also performed in the classical manner, as follows: After pithing the brain a short length of one sciatic nerve was exposed in the thigh, and lifted so that the limb could be ligatured without constricting the nerve. A flap of skin was drawn over the exposed nerve to preserve it from drying. The venom was then injected intraperitoneally. When the animal ceased to respond to external stimuli the spine was cut across, and the sciatic nerves were stimulated on either side of the urostyle.

Experiment 2.

Limnodynastes dorsalis, 18 grammes. Brain pithed. Right sciatic nerve isolated in thigh. Right limb tightly ligated, excluding this. Nerve covered with skin flap. Four mgm. of death adder venom injected intraperitoneally. The frog ceased to respond to external stimuli 1 hour later. The vertebral

column was cut across, the lower limbs and pelvic girdle isolated, and the skin stripped off the hind limbs. The gastrocnemii were stimulated directly, and the sciatics on each side of the urostyle and in the thighs.

	Distance of primary from secondary in cms.	
	Right.	Left.
Gastrocnemii	23	24
Sciatic (urostyle)	26	0 (no contraction)
Sciatic (thigh)	25	0 (no contraction)

Stimulation of the sciatic on the unligated side, though with no separation between the primary and secondary coils there was no contraction of the gastrocnemius, caused vigorous contraction of the muscles of the opposite (ligated) limb. From 5 cm. to 0 cm. there was contraction of the extensor muscles of the thigh on the left (unligated) side.

Performed by either method the experiment gave wholly negative results when *Hyla aurea* was used instead of *Limnodynastes dorsalis*.

Houssay, Negrete, and Mazzocco (1922) have shown that the venom of the tiger snake *(Notechis scutatus)*, among a number of others, has this action. Their experiments suggest that the effect is, in part at all events, a direct one upon muscle. Houssay and Pave (1922) have discussed in detail the poisoning action of venoms on the frog, with special reference to this peripheral action, and give an excellent review of earlier work. They state that the venom of the black snake *(Pseudechis porphyriacus)* also has this action, though Martin (1895), in a careful series of experiments with this venom, was unable to demonstrate it either in rabbits or frogs.

The experiments recorded here were carried out on frogs *(Limnodynastes dorsalis)* ranging from 13 to 21 grammes in weight. The venom was injected intraperitoneally, and the frogs were kept moist under belljars or large funnels. The room temperature was 18–20°C. The results are set out in Table 4, where the time at which paralysis was complete in the hind limbs, and the death time (i.e., the time at which the frog ceased to respond to external stimuli) are recorded, and also the maximal distances in centimetres between the primary and secondary coils at which stimulation of the freshly exposed sciatic nerve and muscle were effective. In the case of frogs injected with the whole venom, or with the soluble fraction, most of the contraction of muscle observed at the close distances recorded for stimulation of the nerve was "overflow," the musculature of the thigh, but not of the calf, contracting from the stimuli.

The curare-like action of the venom is seen to reside wholly in the alcohol soluble fraction of the venom. The insoluble fraction consisted of 18·6% of the total weight of the venom, so that the actual dose equivalent to 4 mgm. of the whole venom was 0·73 mgm. of dry insoluble fraction. With this dose no effect was produced on the sensitiveness of the sciatic nerve or its end-plates. The heart stopped in systole in less than 4 hours. Though weakness of the hind limbs

was observed in these frogs, there was, with the larger doses at least, no true paralysis, and convulsive contractions of the limbs occurred just before death, which was probably due to the action of the venom on the heart.

TABLE 4.

Venom or fraction used.	Weight of frogs in grams.	Dose of whole venom or its equivalent in mgms.	Paralysis time.	Death time.	Number of centimetres at which stimulation effective. For nerve.	For muscle.
Whole	16	4·0	11 mins.	1 hour 7 mins.	3	24
	13	1·0	16 mins.	1 hour 11 mins.	0	24
	14	0·4	22 mins.	56 minutes	5	24
	15	0·2	25 mins.	1 hour 5 mins.	0	20
	15	0·1	1 hour	1 hour 50 mins.	0	26
	14	0·05	2 hours	Less than 12 hours	—	—
Insoluble	16	4·0	—	3 hours 45 mins.	33	24
	21	2·0	—	6 hours 10 mins.	29	13
	19	1·0	—	Between 9 and 12 hours	—	—
Soluble	14	0·2	1 hour	1 hour 44 mins.	6	22
	21	0·1	2 hours	3 hours 20 mins.	0	24
	16	0·05	3¼ hours	3 hours 50 mins.	12	26

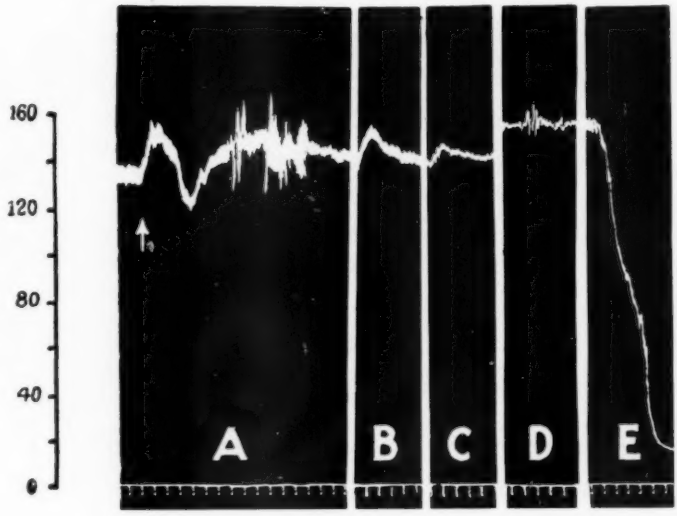

Figure 1.

Tracing of the blood pressure of a cat weighing 2·52 Kilos. At A 4·35 mgm. of insoluble fraction of death adder venom (equivalent to 23·7 mgm. of the whole venom) injected.

B. 23 minutes after injection.
C. 1 hour after the injection.
D. 1 hour and 20 minutes after the injection.
E. 1½ hours after injection.

Time in ½-minute intervals.

The number of animals studied is too small to yield an accurate estimate of the toxicity of the two fractions, but the time required to cause paralysis suggests that there has been some loss of toxicity of the soluble fraction as compared with the whole venom. A dose of the soluble fraction equivalent to 0·05 of the whole venom (0·04 mgm. by weight of soluble fraction) caused a typical curare effect. The heart was beating feebly, and the blood was still fluid when the respiratory movements and the response to external stimuli had ceased. We may reasonably conclude from these results that 0·73 mgm. of the insoluble fraction contains much less of this "curare" activity than 0·04 mgm. of the soluble fraction.

Action of the Fractions on the Blood Pressure of the Cat.

There is present in this venom a substance which causes stoppage of the heart, and with this venom, as Martin (1895) showed to be the case with that of the black snake, artificial ventilation after the failure of respiration does not keep an envenomed animal alive for any considerable period. Though we have not as yet analyzed the action of this venom on the circulation, we record a few experiments in cats, which show a striking difference between the activity of the two fractions. In all these the animals were under full ether anaesthesia, and were allowed to respire ether through a tracheal cannula. The vagi were intact, and the blood pressure was recorded by means of a cannula in one of the carotid arteries.

The fractions were injected intravenously into the femoral vein. Several experiments gave results closely similar to those recorded in Experiments 3 and 4.

Experiment 3. Figure 1. Cat weighing 2·52 kilos.

11.42 a.m.	Blood pressure 140 mm. Hg.
11.55 to 56	4·33 mgm. of insoluble fractions in 13·7 c.cm. saline (equivalent to 23·7 mgm. of whole venom) injected. Blood pressure rises from 132 mm. to 150 and falls to 120 mm.
11.58	Blood pressure 138 mm. Respirations 138 per minute.
12.7 p.m.	„ „ 150 mm. „ 140 „ „
12.14	Blood in cannula clotted—wash out.
12.19	Blood pressure 140 mm. Respirations 144 per minute.
12.43	„ „ 140 mm. Pulse 170. Respirations 140 per minute.
12.57	„ „ 144 mm. Respirations 134 per minute.
1.5	„ „ 150 mm. „ 116 (shallow).
1.10	„ „ 150 mm. „ 104 (shallow).
1.15	„ „ 155 mm. „ 90 (very shallow).
1.18	„ „ 155 mm. „ 74.
	Blood less well aerated. Traube curves.
1.25	Rapid fall of blood pressure, respiration failed, heart still beating and blood fluid. Stimulation of the phrenic nerves with the secondary coil 22 cm. from the primary gave brisk contraction of the diaphragm. Stimulation of the right sciatic nerve gave good contraction of the innervated muscles with a separation of 35 cm. between the coils.

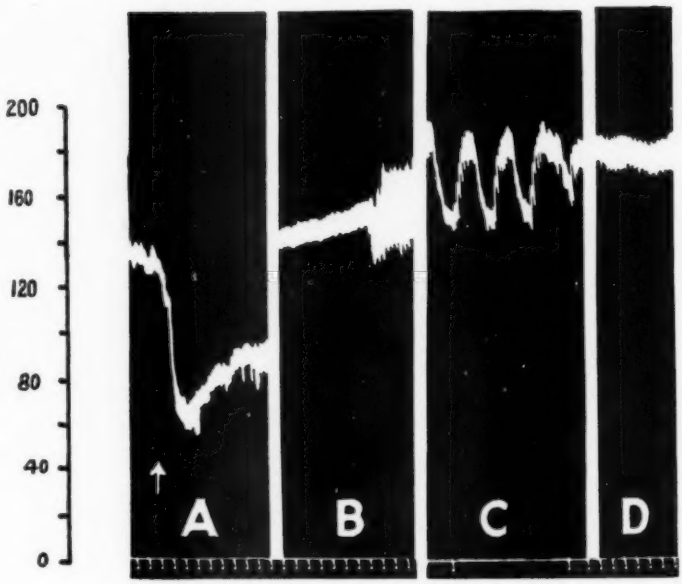

Figure 2.

Tracing of the blood pressure of a cat weighing 3·16 Kilos. At A 4·26 mgm. of soluble fraction (equivalent to 5·2 mgm. of venom) injected.

B. 14 minutes after injection.
C. Drum at faster rate 23 minutes after injection.
D. 25 minutes after injection and 6 minutes before final fall of blood pressure.

Time in ¼-minute intervals

Experiment 4. Figure 2. Cat weighing 3·16 kilos.

2.51 p.m. Blood pressure 128 mm.

2.57 to 2.58 4·26 mgm. of soluble fraction in 3 cm. of saline (equivalent to 5·2 mgm. of death adder venom) injected. Blood pressure falls from 135 to 64 mm. in 1 minute and then rises rapidly.

3.3 Blood pressure 100 mm. Respirations 46 per minute.

3.8 „ „ 120 mm. „ 68 „ „

3.11 „ „ 135 mm. „ 36 „ „

The respirations are now wholly costal and the blood is not so well aerated.

3.15 Blood pressure 150 mm. Respirations 40 per minute. Blood still well aerated.

3.20 Blood pressure 160 mm. Respirations 30 per minute.

3.27 Blood pressure 180 mm. Traube curves. Blood in cannula very dark. Respirations 20 per minute.

3.28 The respiration failed and the blood pressure rapidly fell. The thorax was opened while the heart was still beating. Stimulation of the phrenic nerve gave no contraction of the diaphragm with no separation between the primary and secondary coils. Stimulation of the muscle directly gave a good response with a separation of 25 cm. Stimulation of the right sciatic nerve gave a good contraction of the innervated muscles at 35 cm.

These typical experiments exemplify the striking differences in activity between the two fractions. The soluble fraction causes a sudden fall of blood pressure immediately after injection, the cause of which has not yet been elucidated. A second similar dose in several experiments failed to cause any effect on the blood pressure when this had risen to its original level after the last injection. Death was due to paralysis of the phrenic nerve endings in the diaphragm, an action similar to that described by Brunton and Fayrer (1874) for cobra venom. In none of the experiments with this fraction was there ever any clotting in the cannula.

On the other hand, with the insoluble fraction there was no peripheral, curare-like action on the phrenic endings in the diaphragm. The cause of death was uncertain. Failure of respiration may have been due to central action, as the blood pressure was well maintained throughout the experiments, and the heart was still beating, though feebly, at the end of the experiment. The rapid, shallow respirations are significant, though the anaesthesia in these experiments was kept at fairly light level, the corneal reflex appearing and disappearing throughout. The dramatic immediate fall of blood pressure regularly observed with the soluble fraction was absent following the injection of insoluble fraction. It is noteworthy also that clotting once or twice in the course of these somewhat longer experiments with insoluble fraction always occurred.

DISCUSSION.

The results of the various tests applied to these fractions of death adder venom show that a fairly good quantitative separation of the coagulable proteins from the proteoses has been achieved by a method which does not involve any loss of activity of either fraction. This has permitted a preliminary study of the various activities associated with each of these fractions. The alcohol insoluble fraction has but little of the toxicity of the whole venom when injected subcutaneously in mice or guinea-pigs, and it probably plays no very striking part in causing death unless the venom enters a vein. The feeble coagulant action of the venom in *in vitro* tests is localized in this fraction of the venom. On the other hand, three activities of the whole venom are clearly associated with the alcohol soluble fraction, viz., the curare-like paralysis of the phrenic nerve endings in the diaphragm of some mammals and of the endings in the skeletal muscle of the frog, the immediate fall of blood pressure following intravenous injection in the intact cat and the feeble anticoagulant action of the venom.

On the other hand, this fractionation does not appear to have segregated in either fraction the haemolytic activity of the venom, which appears to be evenly distributed in both fractions. Both fractions are highly toxic by intravenous injection in mice and guinea-pigs, whereas the insoluble fraction has but little

toxicity when injected subcutaneously. On isolated plain muscle (weight for weight) the insoluble has about half the activity of the soluble fraction.

It is possible that the active substances responsible for some of these manifold toxic activities of the venom only represent a very small proportion of the material composing it, and that these are adsorbed to chemically recognizable substances, and are distributed in the fractions by adsorption. This is the more likely, since the separation achieved here is not absolute. The alcohol insoluble fraction contains some proteose, and the soluble fraction some portion of the coagulable protein. The curare-like and the coagulant active principles seem to be sharply localized in opposite fractions, but the methods used for their estimation are possibly not sufficiently accurate to indicate the presence of rather more than a tenth of the activity in the other fraction.

CONCLUSIONS.

1. A method of quantitative fractionation of death adder venom is described by extraction with 45% ethyl alcohol.

2. The method is unattended by any loss of the total activity of the venom.

3. The insoluble fraction has associated with it all the feeble coagulant activity of the venom.

4. The soluble fraction contains the "neurotoxic" principle, which has a curare-like action.

5. The haemolytic activity of the venom is not segregated in either fraction, but appears rather to be evenly distributed.

6. The stimulant action on plain muscle is unevenly distributed between the two fractions, more being present in the soluble than in the insoluble fraction.

7. Titration by subcutaneous injection indicates that almost the whole of the toxic activity recognizable in this way is present in the soluble fraction, but intravenous injection in both mice and guinea-pigs indicates toxicity fairly evenly distributed between the fractions.

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PRELIMINARY COMMUNICATION ON THE INFLUENCE OF AGE OF THE ANIMAL UPON THE NUCLEIC ACID AND COAGULABLE NITROGEN CONTENT OF THE TISSUES OF SHEEP

by

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ANALYTICAL METHODS.

In a recent communication from this laboratory (8), a method of estimating nucleic acid and coagulable nitrogen in tissues is described, which depends upon the following procedures:

(a) Grinding of the tissue with sand, followed by extraction with boiling fiftieth normal alcoholic and aqueous solutions of acetic acid, followed by further extraction with boiling fiftieth normal acetic acid in alcohol, and, finally, with ether, to remove non-coagulable nitrogenous constituents.

(b) Hydrolysis of the residue with normal hydrochloric acid.

(c) Estimation of nitrogen in the hydrolysate and in the non-hydrolysable residue.

(d) Precipitation of purines from the hydrolysate by a modification of Kruger's method.[1]

(e) Oxidation of the purines with permanganate.

(f) Extraction of the oxidation-products with alkaline alcohol.

(g) Estimation of the guanidine derived from guanine by oxidation with permanganate, employing Weber's modification of Marston's colorimetric reagent (6, 9).

[1] By an oversight in the original description of the method the salt employed to buffer the reaction of the mixture from which the purines are precipitated was stated to be trisodium citrate. The salt actually employed is disodium citrate, having, at the dilution employed a pH of 8·3.

The method is being applied in this laboratory in an extensive investigation of the utilization of phosphorus by animals. The data reported in this paper represent the results of a preliminary study of the effects of age upon the nucleic acid content of various organs of the sheep.

Since the publication of the original description of the method the following modifications have been adopted:

Oxidation of the Purine Precipitate.

The procedure of oxidizing the purine precipitate in the centrifuge tube has been abandoned, owing to the difficulty of bringing about complete solution of the impacted precipitate and the danger of loss of material through bumping during boiling with normal hydrochloric acid. Instead, the contents of the tube are now washed out into a small beaker with hot water, the last traces of adherent purine being removed from the sides of the tube with 10 cc. of hot normal hydrochloric acid, which is then added to the contents of the beaker, the residue of acid in the tube being washed into the beaker with a little water. The contents of the beaker are now evaporated to dryness on a water-bath, the residue is taken up in 10 cc. of normal hydrochloric acid, the solution heated to boiling, and the oxidation of the dissolved purines carried out in the manner specified in the original description.

Estimation of the Guanidine yielded by the Oxidation of the Purines.

The final solution of the purine oxidation-products in tenth normal hydrochloric acid is frequently slightly coloured, and the colorimetric readings in such cases might conceivably yield too high an estimate of guanidine. To remove this colour we now employ Kieselguhr, as in the initial decolourization of the hydrolysate. The oxidation-products, after evaporation of the alcoholic extract to dryness, are taken up in 10 cc. of tenth normal hydrochloric acid, with the aid of gentle heating. To the solution, when cool, 0·4 grammes of Kieselguhr is added, and the mixture shaken and allowed to stand for five minutes. It is then filtered in the apparatus figured in the original description of the method, the flask being washed out with three successive lots of 2 cc. each of distilled water, the filter and Kieselguhr being sucked dry between each washing. The filtrates are always colourless. It will be observed that the solution is filtered while acid, instead of being rendered alkaline before filtering, as originally recommended. The filtrate in the 25 cc. volumetric flask is only rendered alkaline immediately before the colorimetric reagent is added. In this way the chance of decomposition of the guanidine in alkaline aqueous solution prior to addition of the colorimetric reagent is avoided. Blank determinations on standard guanidine solutions showed that this revised procedure involved no loss of guanidine.

Correction of the Colorimetric Reading due to Difference of Heights of Columns.

If the height of the column of liquid on the unknown side of the colorimeter is greater than that on the standard side, the concentration of guanidine will be

over-estimated, owing to the colour of the reagent itself.[2] In the method as originally described the attempt was made to correct this error by subtracting from the estimate a quantity proportional to the difference in the heights of the two columns, multiplied by a factor ascertained from estimations of the guanidine content of solutions of known concentration. Further experience has shown us, however, that the necessary correction is subject to a personal equation, so that it is not the same for different analysts, or even for the same analyst on different days. The unknown is now always read against two standards, one above and the other below the strength of the unknown. The correction is then calculated in the following manner:

y = True concentration of unknown.
y_1 = Apparent concentration, estimated against the higher standard.
y_2 = Apparent concentration, estimated against the lower standard.
l_1 = Difference in millimetres of lengths of columns of unknown and standard, employing the higher standard.
l_2 = Difference in millimetres of lengths of columns of unknown and standard, employing the lower standard.
x = Correction per millimetre of difference in lengths of columns.

We have:

$$\frac{y_1}{l_1} = \frac{y}{l_1} + x$$

$$\frac{y_2}{l_2} = \frac{y}{l_2} - x$$

Hence:

$$\frac{y_1}{l_1} + \frac{y_2}{l_2} = \frac{y}{l_1} + \frac{y}{l_2}$$

$$y_1 l_2 + y_2 l_1 = y(l_1 + l_2)$$

$$y = \frac{y_1 l_2 + y_2 l_1}{l_1 + l_2}$$

The true value is therefore found, without the necessity of ascertaining the value of x, by multiplying the apparent concentration read against the higher standard, by the difference between the lengths of columns of the unknown and the lower standard, similarly multiplying the apparent concentration read against the lower standard by the difference between the lengths of columns of the unknown and the higher standard, and dividing the sum of these by the sum of the differences of the lengths of columns.

[2] This difficulty could be overcome by the employment of a colorimeter equipped with the Bürker compensation system. A suitable colorimeter of this type was, however, not available to us during the period covered by the estimations described in this paper.

Determination of the Total (Protein + Nucleic Acid) Nitrogen.

The nitrogen in the hydrolysate is now determined in the following manner, which we find more convenient than the micro-Kjeldahl method:

Twenty-five cc. of the hydrolysate, measured in a standardized pipette, are delivered into a 300 cc. pryex Kjeldahl flask. To this is added 25 cc. of the following reagent:

20 per cent. phosphoric acid: 300 cc.
10 per cent. $CuSO_4 \cdot 5H_2O$: 25 cc.
Sulphuric acid: 500 cc.

The mixture is carefully heated over a micro burner or small bunsen burner flame until white fumes are given off, when a glass funnel is inserted in the neck of the flask, and heating continued until the fluid is colourless or pale green. After cooling and dilution, with the usual precautions, the contents of the Kjeldahl flask are delivered into a 250 cc. volumetric flask, and diluted nearly to the mark with distilled water employed to wash out the Kjeldahl flask. The volumetric flask and contents are allowed to stand until cool, and then diluted to mark and mixed. The silica is allowed to settle for about 24 hours, and 10 cc. of the clear supernatant liquid are then removed with a standard pipette, and introduced into a 250 cc. volumetric flask. The contents are diluted to about 200 cc. with distilled water, and 25 cc. of Nessler's reagent is then delivered into the flask from a pipette, with constant rotation of the contents of the flask. Suitable standards are similarly prepared, 10 cc. of a dilution of 1 in 10 of the hydrolyzing-oxidizing reagent being added to each prior to the Nesslerization. The unknown, unless very close to one of the standards employed, is always read against two standards, one above and the other below the strength of the unknown, and the concentration of the unknown computed in the manner outlined above, so as to eliminate systematic errors and personal equation in the colorimetric readings. The standards usually employed contain 1·0 and 0·5 milligrammes of nitrogen respectively. The estimate thus obtained yields the nitrogen content of 1 cc. of hydrolysate.

The original filter paper, with the contained unhydrolyzed fraction, is placed in another 300 cc. pyrex Kjeldahl flask, and digested with 40 cc. of the following reagent:

85 per cent. phosphoric acid: 300 cc.
5 per cent. $CuSO_4 \cdot 5H_2O$: 50 cc.
Sulphuric acid: 550 cc.

The ignition is carried out as directed above. The contents of the Kjeldahl flask, at the conclusion of ignition, are washed out into a 250 cc. volumetric flask. After cooling and dilution to mark the mixture is filtered, and the nitrogen in 5 cc. of the filtrate is estimated by Nesslerization as described above, the corresponding oxidizing-hydrolyzing reagent being added to the standards. The

estimate thus obtained, multiplied by 50, yields the total nitrogen in the unhydrolyzable fraction.

It will be noted that in neither estimation is the solution neutralized before the addition of Nessler's reagent. There is sufficient alkali in 25 cc. of Nessler's reagent to provide an excess in each case. If the acid in the hydrolyzing-oxidizing mixture is neutralized before Nesslerization the high proportion of salts in the mixture may cause clouding.

Different oxidizing-hydrolyzing reagents are employed for the hydrolysate and the unhydrolyzable fraction for the following reason: The high temperature attained in the mixture made up with 85% phosphoric acid gave rise to an apprehension that some slight loss of ammonia might occur during the prolonged heating required for the completion of ignition. Duplicate estimates of the nitrogen content of hydrolysates failed to demonstrate any loss of nitrogen when the mixture made up with 85% phosphoric acid was substituted for the mixture made up with 20% phosphoric acid. Blanks containing known amounts of ammonium sulphate did, however, show occasional slight losses. It appeared, therefore, advisable to employ the lower proportion of phosphoric acid whenever possible. It is, however, necessary to use the mixture made up with 85% phosphoric acid for the ignition of the unhydrolyzable residue, on account of the high proportion of carbon contained in the filter paper. The unhydrolyzable residues, however, contain only 10% of the total nitrogen in the tissues, so that if any small error should occur in this estimate it would be negligible in proportion to the total. The ignition of the hydrolysate, where any loss would appear in practically the same proportion in the total, can be carried out quite successfully with the mixture made up with 20% phosphoric acid.

PREPARATION OF THE TISSUES FOR ANALYSIS.

Two ewe merino lambs ($W_3$3 and $W_3$4), of which the mothers had been depastured for two years upon grassland top-dressed with superphosphate at the Waite Institute, were brought to the laboratory within 36 hours of birth, weighed, killed with chloroform, and perfused with isotonic saline through the right and left ventricles until the perfusate returned to the auricles was colourless. The various organs enumerated in the tables were then dissected out and weighed, chopped into small pieces with knives, and, where the available bulk of tissue sufficed, 20-gramme samples were immediately ground up with sand and dropped into boiling acidified alcohol (one-fiftieth normal acetic acid). The subsequent steps in the extraction of the tissues were carried out as described in the original communication, except that five alcoholic extractions were employed before the aqueous extractions, instead of two, and, after five extractions with fiftieth normal aqueous acetic acid, the extraction with acidified alcohol was again repeated five times, and, finally, the tissue was extracted from three to five times with ether. The various extracts were collected, and their

phosphorus contents determined, but these estimations are not yet concluded, and the results will be published elsewhere at a later date.[3]

The tissues of both lambs were combined before sampling, so that the analyses represent the mean compositions of the tissues of two animals. The sample of muscle was obtained from the gluteus, and the total weight of muscular tissue was not determined. The skin was carefully removed, and its weight and area determined without dehairing, the weight of wool upon a new-born lamb being negligible in comparison with the weight of the skin. The sample of skin employed for analysis was taken from the belly of only one of the lambs (W_34), and was dehaired with barium sulphide, well washed to remove the sulphide, and then dried between filter papers prior to chopping and sampling.

The small intestines were washed out with a stream of water, drained, and weighed. The weights obtained were therefore approximate, and exceeded the actual weights by the weight of an indeterminate amount of adherent water.

After the removal of the organs enumerated in the accompanying tables, and also the spleens, which were removed for the determination of their manganese content,[4] the residual tissues of the lambs were minced and separately sampled. The sampling of these residual tissues was necessarily approximate on account of the presence of splinters of bone. Nevertheless, the results obtained agree fairly well, as will be seen from the accompanying table, and we may assume that they represent the nucleic acid and coagulable nitrogen content of the residual tissues to within a few per cent. of their true values. The estimates for other organs, excepting the small intestine, are undoubtedly more accurate, while in the case of the small intestine, although the absolute values are subject to an indeterminate error, the ratio of nucleic acid nitrogen to coagulable non-nucleic acid nitrogen (the "nucleo-cytoplasmic ratio") is not less accurately estimated than in the other tissues analyzed.

Six weeks after the lambs were killed for analysis the mother of one of them (W_1232) was similarly killed and perfused, and the various organs were similarly removed and prepared for analysis. The small intestine was not weighed, because its greater bulk rendered washing a longer process, and we were anxious to transfer the tissue to boiling acidified alcohol as quickly as possible to avoid autolysis. No attempt was made to mince and sample the residual tissues after the removal of the specified organs, because the mincing machine at our disposal was not sufficiently powerful to pulverize the bones of an adult sheep.

The weight of the dehaired skin was estimated by Mr. E. W. Lines in the following way: The animal was skinned, care being taken not to stretch the skin at any point. The skin was then spread upon brown paper, and its outline

[3] In the Bulletin series of the Commonwealth Council for Scientific and Industrial Research.

[4] The manganese contents of all of the tissues were determined by Mr. R. G. Thomas. The results of these analyses will appear elsewhere.

traced. The area enclosed by this outline was then determined with the aid of a planimeter. A small sample of skin (from the belly) was then dehaired with barium sulphide, its area separately determined, and its weight, after washing and drying between filter papers, was ascertained. From the proportion which this area bore to the area of the whole skin the total weight of dehaired skin was computed. The weight of wool was similarly estimated from the difference between this computed weight of the skin and the actual weight of the skin plus wool.

RESULTS OF THE ANALYSES.

The results of the analyses are enumerated in Tables 1 and 2. It is clear that in every organ and tissue the nucleo-cytoplasmic ratio, estimated from the ratio of nucleic acid nitrogen to coagulable protein nitrogen, undergoes diminution with age. Previous observers have shown, by microscopic measurements of nuclear and cytoplasmic diameters (1, 4, 7), and by chemical estimations (5), that the nucleo-cytoplasmic ratio falls, at first very rapidly, and later more slowly during the early stages of development. The results herein enumerated show that this fall continues at a diminished rate throughout life.

The relative magnitudes of the nucleo-cytoplasmic ratios obtained from the different tissues correspond very fairly with the anticipations formed from acquaintance with their microscopic structure. The most richly nucleated tissue, Thymus, also yields the highest ratio, and the glandular organs yield much higher ratios than brain and muscle. It is interesting to note that the heart, with short muscle fibres each provided with a nucleus, yields double the ratio found in skeletal muscle in which the fibres are much longer. The value for skin is at first sight somewhat surprisingly high, but it is necessary to remember that the estimate of protein nitrogen, which forms the denominator of the ratio, is based upon the *coagulable* nitrogen content of the tissue. The white fibrous tissue, which is abundant in the skin, and constitutes a species of secretion from the cells which is no longer an integral part of their protoplasm, consists very largely of collagen, which is converted into gelatin and removed from the tissue during extraction with fiftieth normal aqueous acetic acid. The ratio for skin is therefore to be regarded as the ratio corresponding to the cellular elements in the skin.

The fall of the ratios with growth does not, however, occur in the same proportion in every organ, with the result that when the organs are arranged in descending order of their nucleo-cytoplasmic ratios, the succession of organs is different in the adult and the new-born animals. Thus lung and liver, and kidney and skin, exchange places in passing from the lamb to the adult sheep. The reason for this becomes clear only when the changes in the *total content* of nucleic acid and protein nitrogen in the various organs are taken into consideration.

The total nucleic acid in each of the various organs in the lambs and the sheep was computed by multiplying the ascertained percentage in the sample by the total weight of the organ. Since the organs removed from the two lambs were not equal in weight the totals for the two lambs were separately computed.

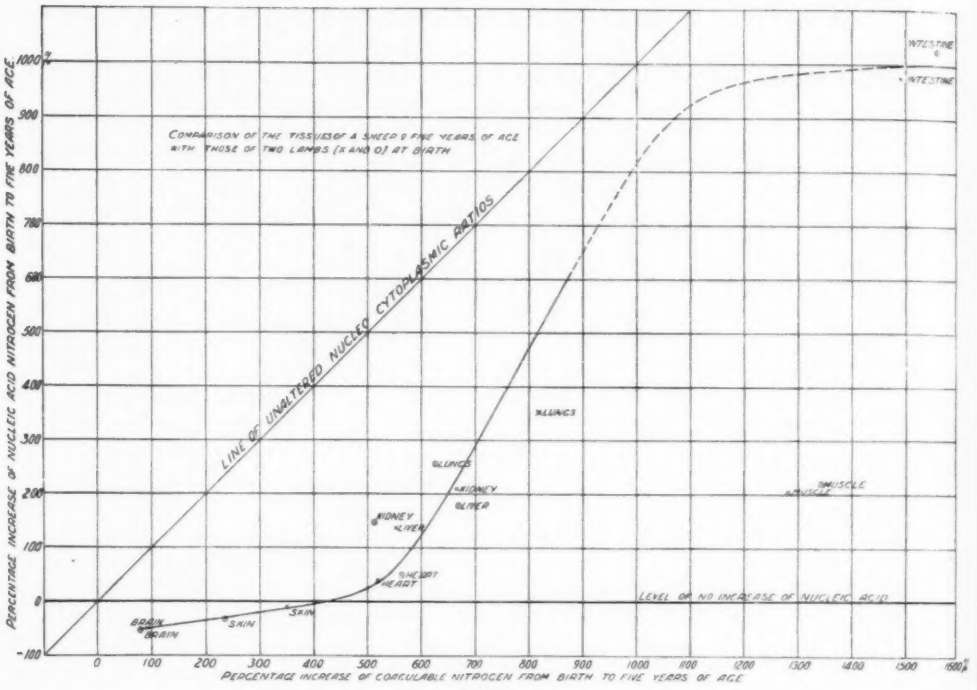

Fig. 1.

When this computation is carried out it becomes evident that the total content of nucleic acid in intestine, liver, lung, kidney, heart, and muscular tissue increases, as might have been expected, with the growth of the animal. The nucleic acid content of the brain (cerebrum), on the contrary, falls to 50% of its value during growth from birth to five years of age, while the nucleic acid content of the skin similarly undergoes a diminution of about 20%. At first sight these facts constituted a puzzling anomaly, but on similarly computing the change in the total protein content of the various organs with age it became evident that so far from constituting an anomaly, the loss of nucleic acid in brain and skin during growth is a necessary consequence of an interdependence of nucleic acid growth and protein growth which has not hitherto been susceptible of demonstration.

In the accompanying figure (figure 1) the alterations of total nucleic acid nitrogen in the various organs during growth from birth to five years of age are represented as percentages of the amounts present in the corresponding organs of the lamb, and form the ordinates of the curve. The abscissae are the corresponding percentage alterations of the total coagulable nitrogen contents of the organs. Since the weights of the organs in the two lambs were not identical the percentages are separately calculated upon the basis of the different amounts of the materials found in the organs of the two lambs.

In the cases of the muscular tissues, which were not weighed, and of small intestine, which was only approximately weighed in the lambs, the total amounts of nucleic acid and protein could not be ascertained. *The ratio* of the amounts in the ewe and lambs, and consequently the percentage increases of each with growth, were computed on the assumption that the growth of these tissues, from birth to five years of age, was proportional to the increase of body weight. This assumption would be approximately correct in the rat (2), but we have no proof of its correctness in the sheep. It fortunately happens, however, that the position of these tissues upon the chart in figure 1 is such that very large errors may be incurred without affecting the conclusions derived from inspection of the curve. Thus to bring the points representing muscular tissue into the proximity of the curve the ratio of the weight of muscular tissues in the adult to their weight in the lambs must have been no less than 60% under-estimated. Such an extent of under-estimation, when we consider what a large proportion the muscular tissues constitute in the weight of an animal, is clearly impossible. Similarly the ratio of the weight of the adult small intestine to the weight of the small intestine in the lamb, may be 30% in error, above or below the assumed ratio, without affecting the essentially sigmoid character of the curve.[5] However, in view of the uncertainty attaching to the precise position of the points representing the intestine, and the absence of points lying between the lungs and the intestine, the upper portion of curve 1 is drawn as a broken line. The rearrangement of the data in figure 2, however, brings the points representing the intestine into closer proximity to the other points on the curve, and it becomes evident that the true values for intestine cannot be very far from the estimates based upon proportional growth of body and intestine.

It is at once clear from the diagram in figure 1, and the curve which is drawn between the ascertained points, that all of the tissues and organs analyzed, excepting muscular tissue, fall upon a sigmoid curve, which descends below the line of zero increase of nucleic acid nitrogen at 420% increase of protein nitrogen. In other words, there is a definite quantitative relationship between the rate of growth of cytoplasm (represented by protein) and the rate of growth

[5] Whatever the true weights of these tissues may have been the points must lie upon a straight line passing through the assumed estimates and parallel to the "line of unaltered nucleo-cytoplasmic ratios".

of nucleus (represented by nucleic acid), which is of such a character that if the rate of growth of cytoplasm is not maintained above a level corresponding to 420% increase in five years, the nucleic acid content of the organ must necessarily undergo retrogression with age instead of increase. Brain and skin are therefore no longer anomalous, and the only anomaly among the organs studied is now seen to be afforded by muscular tissue, a fact which would hardly have been suspected from a casual inspection of the analytical results.

The anomalous position of muscular tissue in the diagram is very suggestive, because this is the only tissue, excepting thymus, which contains extra-nuclear nucleic acid. The extra-nuclear nucleic acid (inosinic acid) in muscular tissue would not be included in our estimates, because it is a mononucleotide of hypoxanthine, while our method of analysis only determines nucleotides of guanine, which are the characteristic nucleotides of intra-nuclear nucleic acids. This fact suggests the possibility that muscular tissue may not in reality be so anomalous as it appears, and that the functional relationship of nucleic acid to protein which our figures reveal is in this tissue partially undertaken by an extra-nuclear nucleic acid, inosinic acid. The enormous hypertrophy of muscle-cells which accompanies their growth tends, in fact, to reduce the proportion of visible nuclear material to an exceptionally low level, and the necessity may thus arise of providing an extra-nuclear source of those functions which the nucleic acid of the nucleus normally performs. This would provide a reason, hitherto lacking, for the existence of substantial amounts of an extra-nuclear nucleic acid in this particular tissue and no other adult tissue. The apparently anomalous position of muscular tissues in the graph, therefore, substantiates, rather than otherwise, the interdependence of nucleic acid and protein synthesis which our data have revealed.

The relationship between nuclear and protein synthesis is evidently not a linear one, but of such a character that each increase of the rate of protein synthesis, up to a certain limit, demands a more than proportional increase of nuclear synthesis. In other words, the synthesis of nucleic acid undergoes catalysis relatively to that of protein. This corresponds with the observation of morphologists that the growth of the nucleus, during the interdivisional stages, undergoes acceleration relatively to the growth of the cytoplasm (7).

The disproportionate acceleration of nucleic acid synthesis with increase in the rate of protein synthesis is also the origin of the unequal fall of nucleo-cytoplasmic ratios in different organs, to which attention was drawn above. The nearer the various points in figure 1 lie to the "line of unaltered nucleo-cytoplasmic ratios" the less will be the fall of the ratio with growth. In consequence of the sigmoid character of the relationship between nucleic acid and protein synthesis, it necessarily follows that the points representative of the various organs lie at unequal distances from the "line of unaltered nucleo-cytoplasmic ratios." From the diagram one can therefore perceive at a glance

the relative decrease of the nucleo-cytoplasmic ratios in the different organs studied during growth from birth to five years of age.

We are indebted to our colleague, Professor H. H. Woollard, for drawing our attention to the fact that the order of increase of nucleic acid and protein in the various organs studied is approximately the inverse order of their assumption of physiological activity during development. This is more clearly

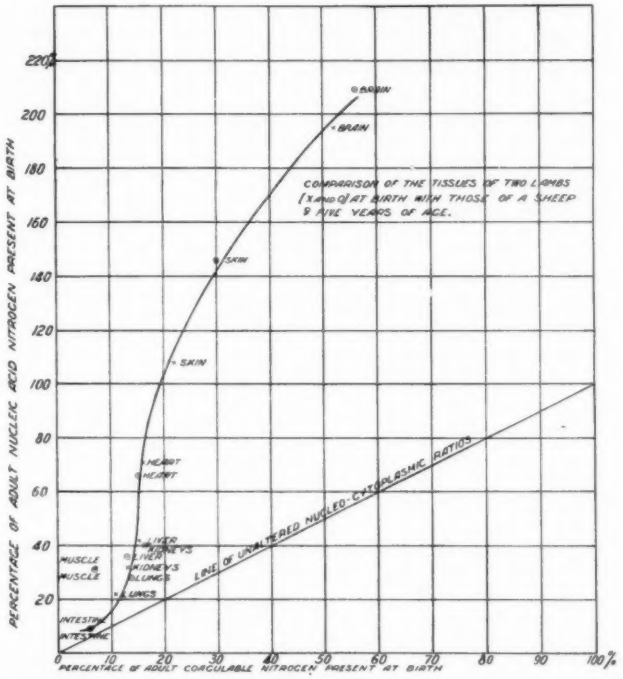

Fig. 2.

evident in the diagram in figure 2, which we constructed after receiving this suggestion. In this diagram the ordinates represent the proportion of the adult nucleic acid contents of the organs which had already been synthesized at birth, and the abscissae similarly represent the proportion of the adult protein content which had been synthesized at birth. It is clear that brain, which enters upon its functional activity very early, has nearly completed its chemical development at birth. Skin, which is also developed early, has similarly achieved a large proportion of its chemical development. Lungs and intestine, which have not begun to function, have only accomplished a small proportion of the chemical development which they are destined to undergo.

SUMMARY.

1. The nucleic acid nitrogen and coagulable nitrogen contents of various organs in the new-born lamb and adult sheep have been determined.

2. In every organ and tissue examined the nucleo-cytoplasmic ratio falls considerably with age.

3. The fall of the nucleo-cytoplasmic ratio with age is not the same in different organs.

4. The total amount of nucleic acid in the adult cerebrum is 50% less than the total amount in the cerebrum of the new-born lamb. The total amount of nucleic acid in the skin is also diminished with growth. The total nucleic acid content of the other organs studied was greater in the adult than in the lambs.

5. If the percentage increases of total nucleic acid content of the various organs with growth from birth to five years of age are plotted as ordinates against the corresponding percentage increases of protein (coagulable nitrogen) content as abscissae, the resulting curve is sigmoid, and indicates that the synthesis of nucleic acid is catalyzed relatively to that of protein.

6. The only anomalous tissue among those investigated is skeletal muscle. It is suggested that the anomalous position of this tissue is attributable to the fact that it contains an extra-nuclear nucleic acid (inosinic acid), which would escape estimation by our methods of analysis, and which discharges certain essential functions of nucleic acid, which, in other tissues, is confined to the nucleus.

7. Our results reveal an essential interdependence of nucleic acid and protein synthesis in animal tissues.

ACKNOWLEDGMENTS.

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ANALYSIS OF TISSUES OF LAMBS.

$W_3 3$, ♀, Weight, 3·94 kg.; $W_3 4$, ♀, Weight, 3·775 kg.

Organ.	Weight of organ. Grams. Lamb $W_3 3$	Lamb $W_3 4$	Solids in organ. Per cent.	Coagulable nitrogen. Weight of sample. Grams.	Extracted residue in sample. Per cent.	Hydrolysate. Mgms.	Unhydrolysable residue. Mgms.	Total in sample. Mgms.	Per cent. in residue.	Total in organ. Grams. Lamb. $W_3 3$	Lamb. $W_3 4$	Nucleic acid nitrogen. Total in Sample. Mgms.	Per cent. in residue.	Total in organ. Mgms. Lamb $W_3 3$	Lamb $W_3 4$	Protein + Nucleic Acid. Per cent. in organ.	Per cent. in residue.	Nucleo-Cytoplasmic Ratio.
Thymus	24·5	13·0	16·6	20·25	11·7	345	23	368	15·5	·445	·236	59·9	2·53	72·5	38·4	11·4	97	·194
Small intestine			11·0	20·00	7·75	209·5	17·5	227	14·65			14·6	·945			7·1	92	·069
„				20·00	7·75	207	11	218	14·1			15·7	1·014			6·8	88	·078
Liver	129·9	110·4	17·95	20·24	11·8	300	22·5	322·5	13·4	2·17	1·84	19·9	·828	132	112	10·0	84	·066
„				19·85	12·1	321	26	347	14·45			20·8	·868			10·9	86	·064
Lungs	70·3	88·9	16·2	20·00	9·25	272	20·5	292·5	15·8	1·02	1·29	17·1	·923	59·9	75·7	9·1	99	·062
„				20·00	9·83	269	19	288	14·65			17·0	·865			9·0	92	·063
Skin	580	780	$W_3 4$ 18·1	20·30	11·2	335	25	360	15·85	9·15	12·31	19·2	·845	474	638	11·2	99	·056
„				20·27	9·03	249	32	281	15·3			14·0	·764			9·0	96	·052
Kidneys	21·8	27·3	15·4	15·46	9·54	205	18	223	15·2	·318	·398	10.4	·705	15·3	19·2	9·0	95	·049
„				15·05	9·61	202	19	221	15·3			11·0	·763			9·2	96	·053
Cerebrum	44·5	47·5	15·4	40·25	7·68	425	52	477	15·45	·533	·569	12·8	·415	14·2	15·2	7·4	96·5	·0276
„				20·30	7·68	224	24	248	15·9			6·55	·420			7·6	100	·0271
Heart	34·1	31·8	21·1	20·00	12·4	358	31	389	15·7	·672	·627	8·60	·346	15·1	14·1	12·2	98	·0226
„				20·00	13·2	366	33	399	15·1			9·15	·346			12·5	95	·0235
Muscle			18·0	20·00	12·4	317	28	345	13·9			4·88	·196			10.8	87	·0143
„				20·00	16·1	366	32	398	12·35			6·56	·204			12·4	77	·0168
Residual tissues $W_3 3$			24·2	25·14	15·6	406	19	425	10·8			6·17	·157			10.6	68	·0147
$W_3 4$			26·3	25·05	16·7	445	22	467	11·1			7·32	·174			11·7	70	·0159

ANALYSIS OF TISSUES OF ADULT SHEEP.

(♀ Aet. 5 years.)

$W_1$232; Total weight, 43·6 kg.; Wool, approx. 3·6 kg.

Organ.	Weight of organ. Grams.	Solids in organ. Per cent.	Coagulable nitrogen.							Nucleic acid nitrogen.			Protein + nucleic acid.		
			Weight of sample. Grams.	Extracted residue in sample. Per cent.	Hydrolysate. Mgms.	Unhydrosylable residue. Mgms.	Total in sample. Mgms.	Per cent. in residue.	Total in organ. Grams	Total in sample. Mgms.	Per cent. in residue.	Total in organ. Mgms.	Per cent. in organ.	Per cent. in residue.	Nucleo-cytoplasmic ratio.
Small intestine		14·4	20·00	10·5	304	16	320	15·2		14·8	·701		10·0	95	·0484
„			20·00	10·8	303	17	320	14·8					10·0	93	
Lungs	471·6	19·1	20·00	12·1	374	26	400	16·5	9·36	12·3	·506	271	12·5	103	·0316
„			20·00	12·3	370	25	395	16·0		10·8	·438		12·3	100	·0281
Liver	669·4	24·2	19·75	14·6	375	38	413	14·4	14·17	9·05	·314	315	13·1	90	·0224
„			19·93	14·8	387	40	427	14·5		9·61	·327		13·4	91	·0230
Kidneys	137·3	17·5	20·28	11·8	320	32	352	14·7	2·43	6·99	·292	47·8	10·8	92	·0203
„			20·04	12·1	331	32	363	14·9		7·06	·290		11·3	93	·0198
Skin	1700	24·5	20·01	14·7	472	17	489	16·7	41·22	5·30	·181	437	15·3	104	·0110
„			20·22	15·3	472	15	487	15·7		5·04	·163		15·0	98	·0105
Cerebrum	75·2	22·7	20·28	8·6	243	32	275	15·8	1·020	1·965	·113	7·3	8·5	98	·0072
Heart	216·1	19·0	20·00	12·2	368	30	398	16·4	4·16	3·94*	·080	21·3	12·5	102	·00514
„			20·00	12·3	343	29	372	15·1					11·6	94	
Muscle		23·2	20·00	17·4	451	43	494	14·2		3·19*	·047		15·4	88	·00345
„			20·00	16·7	395	39	434	13·0					13·6	81	

*Total in both samples.

OBSERVATIONS ON A PERMANENTLY LYSOGENIC STRAIN OF *B. ENTERITIDIS GAERTNER*

by

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The most disputed points in the current controversies as to the nature of bacteriophage are probably (1) the possibility of procuring phage from normal bacteria and (2) the status of permanently lysogenic bacteria. The two subjects are obviously closely related. According to some writers there is no sharp line to be drawn between normal and lysogenic strains, all bacteria being potentially lysogenic, and differing only in the ease with which a liberated phage can be recognized. In his most recent discussion of phage theory Bordet (1928) lays great stress on the significance of naturally lysogenic strains, which he regards as primitive forms, producing more or less accidentally a principle which, innocuous to the producing strain, provokes typical bacteriophage lysis when it comes into contact with certain other more sensitive bacteria. The present note is concerned with one of these naturally and permanently lysogenic strains of bacteria.

The strain (B.T.M.) has been fully described previously (Burnet, 1929). It was received as a rough motile strain of *B. enteritidis*, showing the typical antigenic constitution of such strains, though in its physical characteristics less typically "rough" than usual. By methods which have been described previously, a large range of variants was derived from this culture, including forms with the four chief antigenic structures smooth motile (HO), smooth non-motile (O), rough motile (HR), and rough non-motile (R).

The original HR form produced in broth cultures a phage that could be detected by its action on a smooth strain of *B. sanguinarium* (NCTC 398S). In most experiments a 24-hour broth culture of the lysogenic strain was heated to 57° for 30 minutes, and the heated culture was then titrated by spreading known dilutions on an agar plate previously inoculated with *B. sanguinarium*. Perfectly typical bacteriophage lysis occurred, and the phage produced could be carried on indefinitely in broth cultures of strain 398S.

If a single colony of the original strain was added to a tube of broth, and incubated for 24 hours, titration showed approximately 2×10^6 plaques per c.cm.

The smooth derivatives were considerably less active, showing roughly 2–3,000 plaques per c.cm. These estimations were made in England in 1927. Eighteen months later a fresh series of experiments was commenced in Australia. Despite numerous subcultures the two types still showed the same general activity.

The four stock cultures (HO, HR, O, and R) were replated from broth. The HO plate showed some irregularities amongst the colonies, and five more or less distinguishable types were picked off. The other plates appeared uniform and two colonies of each were isolated. Amongst the cultures from HO, one was a rough non-motile variant, and one showed open lysogenicity by producing nibbled colonies. These were discarded. All the other cultures showed permanent characters of the type to be expected from their antigenic constitution.

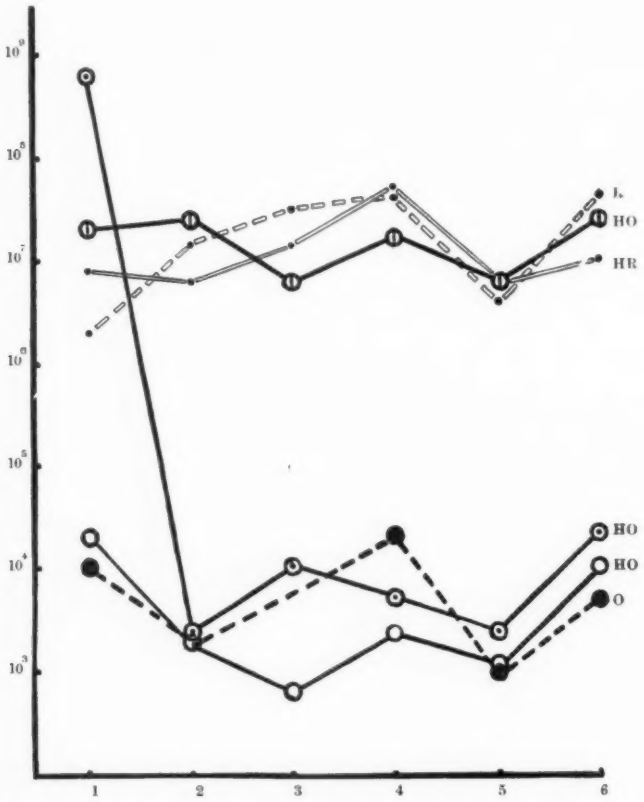

Figure 1. The production of phage by successive generations of the various antigenic types of strain B.T.M. The number of phage particles per c.cm. of a 24-hour broth culture is shown for six successive isolations from single colonies of six variants of the antigenic constitutions shown.

The lysogenic power of seven such cultiures (three HO forms, two O forms, and one each of HR and R) was tested for six successive generations. An isolated colony was picked off into broth. From this a fresh plating was made immediately, and the broth tube then incubated for 20 hours. The culture was then heated to 55° for 30 minutes, and titrated on plates spread with *B. sanguinarium*. The process was then repeated with each replating.

The successive titrations are combined in figure 1, which shows the number of plaques per c.cm. produced at each generation. The two O strains behaved in almost identical fashion, and one is omitted to avoid choking the figure.

It can be seen at once that the cultures fall into two groups. The rough strains and one smooth motile strain produce around 10^7 plaques per c.cm. at each generation. The bulk of the smooth strains give rise to only a few thousand plaques per c.cm. The findings are practically constant at each isolation.

The aberrant behaviour of the strain HO_3 is clearly due to the fact that the first colony tested was in an unstable actively lysogenic state. Subsequent generations were composed of organisms in the stable condition. The actively lysogenic nibbled colony included in the first isolation and discarded had a still higher titre, showing over 10^{10} plaques per c.cm.

In addition to the four classical antigenic varieties described, an extensive series of other variants of this strain was tested for lysogenic power. The following strains gave in broth culture titres around 10^7 per c.cm.:

(a) Eight specifically resistant rough strains derived by the action of various phages.
(b) A cohesive variant, giving tough, dry, wrinkled, colonies on agar, and forming a pellicle in broth, antigenically HR.
(c) A mucoid variant antigenically R.
(d) Another mucoid variant showing a secondary lysogenic power (see below).

The following showed the lower degree of lysogenesis (10^3 to 10^4 plaques per c.cm.):

(e) Two smooth specifically resistant variants produced by phage action.
(f) A cohesive pellicle-forming variant antigenically HO.
(g) Smooth HO and O forms recovered after animal passage.

The strains tested therefore include practically every variant type that has ever been described amongst the Salmonella group. Every single colony that has been tested has shown definite lysogenic activity. It is evident that the lysogenic function in this case is characteristic of every cell in the culture, and presumably is intimately related to some of the most permanent of the cell's activities.

The Liberation of Phage from a Lysogenic Culture.

The phage produced by this strain can be propagated only at the expense of certain smooth Salmonella strains. It has never shown the slightest activity on any rough culture. Yet the rough form of BTM produces much more of the phage than the average smooth form. This suggests at once that the liberation of phage is by some totally different process from that occurring in ordinary lysis.

There is not much work reported on this subject. Gildemeister and Herzberg (1924) found with their strain Coli 88 that phage was produced more or less parallel with the growth of the culture, except at low temperatures. At 9°C. growth of the organism took place, but no liberation of phage. Flu (1925), however, working with the same strain, found that phage was produced to some degree under all conditions that allowed growth of the lysogenic strain to occur. Both would agree that any phage production that occurs takes place during bacterial multiplication. The following experiments show that this is not necessarily the case with the lysogenic strain BTM–HR.

Experiment 1. Two agar plate cultures of BTM–HR, grown for 20 hours at 37°, were scraped off with a glass rod and emulsified in 12 c.cm. of normal saline. Five c.cm. of the emulsion were measured into each of two centrifuge tubes, and the tubes centrifuged for 20 minutes at about 2,500 revolutions. The supernatant fluid was removed and set aside for titration. The deposit, drained as completely as possible of fluid, was then re-emulsified in 5 c.cm. of the liquid to be tested, which had been previously warmed to 37°. The mixtures were placed in the incubator for half an hour, and were stirred at intervals. They were then centrifuged for 20 minutes, the supernatant fluid put aside for titration, and the whole process repeated as often as required.

The supernatant fluids as they were obtained were heated to 55° in a water-bath for 30 minutes, cooled rapidly, and titrated in duplicate by plating suitable dilutions on agar, spread with the sensitive indicator strain 398S. Plaque counts were made on the following day. The results of two experiments, in which distilled water, 2% sodium chloride solution, and 2% calcium chloride solution were used, are tabulated. The figures are calculated from the average of two counts at the most convenient dilution. The duplicates agreed in nearly all cases to within 10%.

TABLE 1.

Showing number of phage particles per c.cm. liberated at successive washings of BTM–HR.

		A.		
1st washing	Normal saline	16,700,000		
2nd "	Distilled water	9,760,000	2% saline	4,300,000
3rd "	"	2,510,000	"	750,000
4th "	"	521,000	"	708,000
5th "	"	148,000	"	751,000

B.

1st washing	Normal saline	56,800,000		
2nd „	2% saline	2,290,000	2% $CaCl_2$	4,590,000
3rd „	„	1,540,000	„	2,466,000
4th „	„	418,000	„	609,000
5th „	Distilled water	4,100,000	Distilled water	1,620,000

Somewhat larger amounts of phage were liberated when the bacteria were washed in the same way with broth, but since in these experiments there was a possibility of multiplication occurring no stress can be laid on the finding. But the findings tabulated show unequivocally that phage can be liberated in considerable amount by resting bacteria in non-nutritive media. An examination of the table, particularly of Experiment B, suggests that there is only a limited proportion of the bacteria present that can liberate phage under these conditions. Omitting the first washing from consideration, the four subsequent washings gave in each case between 40 and 50 million particles. The smaller initial liberation by the sodium chloride solution was balanced by the larger yield with the final washing in distilled water.

If the phage particles are pre-existent within the lysogenic bacteria one might expect them to be liberated when lysis by some unrelated phage occurred. It was possible to investigate this with the strain BTM–R, since a phage (No. 13) was available, capable of lysing this strain, but completely without action on the indicator strain, *B. sanguinarium*, used to detect the homologous phage.

Experiment 2. The following cultures were grown in broth:

(a) BTM–R alone.
(b) BTM–R plus a loopful of phage 13.
(c) A rough non-lysogenic strain (Wa R), also sensitive to phage 13, plus a loopful of this phage.

These were incubated overnight, and then heated to 55° for half an hour. They were then titrated for phage content on a rough strain sensitive to phage 13, and on the smooth *B. sanguinarium*. The titres found on the smooth strain were:

(a) BTM–R alone: 2,200,000 particles.
(b) BTM–R + phage 13: 530,000 particles.
(c) Wa R + phage 13: Nil.

Both (b) and (c) showed the presence of phage 13 in its usual amount.

Lysis by an unrelated phage thus diminishes the amount of homologous phage liberated, and there is no evidence that the intrinsic phage exists within the lysogenic bacteria as an independent unit.

Amongst the resistant colonies obtained after lysis of strain BTM–R by phage 13 was a mucoid lysogenic variant. If this was subcultured directly on McConkey's medium it remained fairly stable, but when transferred to broth and plated after incubation gave a mixture of smooth and rough colonies. The broth

contained a large amount of phage 13, and when titrated on *B. sanguinarium* showed 1,900,000 particles per c.cm. The mucoid colonies were thus doubly lysogenic, but the coexistence of another lysogenic function made no appreciable difference to the "normal" lysogenic activity.

DISCUSSION.

The chief interest of the present observations concerns the rather striking differences in the production of phage on the one hand by permanently lysogenic strains, and on the other by normal strains under the influence of extrinsic lysis.

The strain described maintains its lysogenic power unaffected through almost all the variations of antigenic structure and mode of growth possible for its species. If the lysogenic power is quantitatively estimated the variants fall into two groups. All the rough strains tested and one smooth culture produced a large amount of phage (around 10^7 particles per c.cm.). The rest of the smooth strains all gave a few thousand particles per c.cm., with the exception of one culture, which was unstable, and showed evidence of frank lysogenesis (nibbled colonies). This naturally gave a very high titre of liberated phage. When the specific phage involved is tested for its action on a wide range of bacteria it is found to lyse actively, smooth *sanguinarium* and *pullorum* strains, and less regularly many smooth *typhosus* and *enteritidis* cultures. No derivative of the phage has ever shown the slightest action on any rough strain.

If we consider only the smooth phase the strain behaves exactly as the lysogenic coli strain L, originally described by Lisbonne and Carrère (1922), and extensively studied by Bordet and Renaux (1928). There is a more or less standard degree of lysogenesis, but individual colonies vary considerably, some producing so much phage as to cause frank lysis of part of the culture. Such results are compatible with the view that there is an unstably balanced struggle between phage and bacterium, a very small proportion of the bacteria always undergoing lysis of the ordinary type. But the relatively high lysogenic capacity of rough strains cannot be explained on this view, since the phage produced is incapable of lysing any rough cultures. The liberation of phage here must be determined entirely by internal factors uninfluenced by the presence of phage in the medium. It is a natural corollary from this to assume that the phage production of smooth cultures in the stable state is also a manifestation of internal conditions, rather than the result of a balanced external antagonism.

The permanence of the lysogenic character makes it necessary to assume the presence of bacteriophage or its *anlage* in every cell of the culture, i.e., it is part of the hereditary constitution of the strain. The conditions under which this *anlage* is liberated or activated are extremely obscure. The experiments described above, in which phage was obtained by washing bacteria with non-nutrient solutions, show that the liberation is not necessarily associated with growth. Distilled

water was particularly effective in liberating phage, even after the bacteria had been four times washed with saline solutions. This suggests that the phage particles are preformed within the bacteria, and diffuse out when the bacteria are swollen or disrupted by imbibition of water. Against this view is the fact that no excess of the intrinsic phage is liberated when the bacteria are lysed by an unrelated extrinsic phage. The numbers of bacteria used in the washing experiments were, however, very much greater than those involved in the second type of experiment, and it is likely that only a small percentage of the bacteria (about 0·1% from the data available) are capable of liberating phage when treated with distilled water. Even if all this was liberated by extrinsic lysis the experimental methods available would probably fail to show any definite increase.

The conclusion we arrive at, therefore, is somewhat as follows: All bacteria of this permanently lysogenic type contain in their hereditary constitution a unit potentially capable of liberating phage. In a proportion (perhaps about 0·1% in day-old agar cultures) phage is actually present within the bacteria, and can be liberated by treatment with distilled water. Phage may also be liberated, presumably from bacteria of this group, during the normal processes of growth. The evidence further suggests that unless the activation of the hereditable *anlage* takes place spontaneously, disruption of the cell by any means will not liberate phage. The essentials of the process seem to take place entirely within the cell. There seems to be no evidence available as to what determines whether or not intracellular activation will occur. The conditions are probably analogous to those concerned in such inorganic processes as monomolecular reactions or radioactive disintegrations.

These observations are in entire accord with the view put forward by Bordet and Renaux, that in these cases the lytic principle (or its *anlage*) is a normal (i.e., physiologically co-ordinated) constituent of the bacterium, which spontaneously secretes it without injury to itself. But as Bordet and Renaux also point out, its must not be assumed from this that the ordinary process of phage multiplication on sensitive bacteria is of the same nature.

Liberation of phage from a permanently lysogenic strain depends entirely on intracellular changes; it is essentially uninfluenced by changes in the antigenic structure of the strain or by lysis with extrinsic phages. In a strictly limited sense it is possible to liberate phage in considerable amount from bacteria that are not growing.

Classical bacteriophage lysis, on the other hand, is primarily dependent on the nature of the phage used, it is greatly influenced by changes in the antigenic structure of the bacterial strain, and liberation of phage can only be obtained with actively growing bacteria.

In the first case we have the bacteriophage unit physiologically co-ordinated in the hereditary constitution of the bacterial strain, but liberated into the medium under certain obscure conditions. Toward a sensitive bacterium this

same unit behaves as a predatory entity, which becomes specifically adsorbed to the surface, and multiplies genetically within the bacterial cell, which it eventually destroys.

The difficulty of reconciling these two aspects of bacteriophage phenomena has been responsible for all the current controversy on the intimate nature of phage, whether it is an independent parasite or a pathologically altered constituent of normal bacteria. In our view both these contentions have been completely proved, and the current attitude on both sides of regarding them as irreconcilable alternatives is quite unjustified. According to the particular type of bacterium that is reacting with the phage concerned, it may be useful and convenient to regard the phage as an independent parasite or as a unit liberated from the hereditary constitution of some bacterium, the usage being determined wholly by its functional activity at the time.

SUMMARY.

1. A permanently lysogenic strain of *B. enteritidis* Gaertner is described.

2. Practically every known variant of this species was tested, and all showed characteristic lysogenic activity.

3. Rough strains liberated more phage than the majority of smooth cultures.

4. Successive amounts of phage can be liberated from washed lysogenic bacteria in the presumed absence of bacterial multiplication.

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A CHEMICAL AND HISTOLOGICAL INVESTIGATION IN VICTORIA (AUSTRALIA) OF THE BLOOD OF CATTLE AND SHEEP

by

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During the past few years certain diseases of cattle and sheep have been investigated at the Veterinary Research Institute, Melbourne University. In many cases the usual pathological and bacteriological examinations failed to demonstrate the etiology, and it has been felt that useful information might be gained by an analysis of the blood of affected animals.

Unfortunately there are no standards of comparison, as at the present time little or no information is available on the constituents of the normal blood of sheep and cattle in this country, and the investigations here described were undertaken to remedy this lack of knowledge, and to form a ground-work on which further research might be based.

Although similar work has been done elsewhere, the want of uniformity in the results relating to animals in different countries suggests strongly that local conditions may be important factors.

SOURCE OF MATERIAL.

The blood samples were taken from sheep and cattle slaughtered at the Melbourne City Abattoirs, Flemington, Victoria. In addition to Victorian animals these would include sheep from the Southern Riverina, New South Wales, and a certain number of cattle from the other States. The samples were collected at random from the animals as they were killed, no attempt being made to select special subjects for examination. It was found impracticable to differentiate between healthy and diseased subjects, or to trace the subsequent history of each donor, but it is assumed that the majority of the animals could be regarded as in average health.

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[1] The biochemical investigations were carried out by Joan H. Norris and the histological investigations by W. E. Chamberlin.

METHODS.

In this investigation the blood was examined both chemically and histologically. The blood was allowed to flow from the throat of the animal into a clean glass container, and transferred immediately to small stoppered, glass phials, containing different anticoagulants (see later). Smears for histological examination were made from fresh blood at the same time.

CHEMICAL METHODS.

Estimations have been made of the following blood constituents: Sugar, calcium, urea, non-protein nitrogen, uric acid, creatinine, and inorganic phosphate. In some cases the moisture content has been determined.

Particulars of the methods adopted are given in brief, with the references necessary for complete details.

(a) *Sugar.* A special anticoagulant and preservative was used for the blood for sugar estimations. The blood sugar is known to disappear fairly rapidly if the blood is allowed to stand for a few hours, and, as it was usually not until three hours after being collected that the sugar estimations could be done, a preservative was added, consisting of 1 mgm. of a mixture of sodium fluoride and thymol, in the ratio of 5 : 1, to 1 cc. blood, as used by Splatt (1). An hourly series of blood sugar estimations, carried out during six hours to test the efficacy of the mixture, showed that glycolysis was negligible over that period.

The method for determining the sugar was that of MacLean (2).

(b) *Calcium.* The calcium was estimated by precipitation from serum with saturated ammonium oxalate (3). The precipitate was washed, dissolved in acid, and titrated with N/100 potassium permanganate, freshly prepared each time from a stock N/10 solution.

(c) *Urea.* The anticoagulant used in this, and in all other estimations, was lithium oxalate, added by the cotton gauze method recommended by Folin (4). The urea was determined by MacLean's soya bean method (5).

(d) *Inorganic Phosphate.* This was estimated in the protein-free filtrate, prepared with trichloracetic acid, by the colorimetric method of Fiske and Subbarow (6). This method, which has been found very satisfactory, consists in the addition of ammonium molybdate and subsequent reduction, in the presence of sulphite, with 1, 2, 4 aminonaphthol sulphonic acid, instead of hydroquinone, which has been the most used hitherto. As it could not be obtained in any other way, this acid was made according to the directions of (7).

The remainder of the investigations, with the exception of the moisture, were made on a protein-free filtrate prepared according to Folin's method (4). Difficulty was experienced in completely removing the protein with the quantities of reagents advised by Folin, i.e., with 10 cc. blood were used 70 cc. of distilled water, 10 cc. of 10% sodium tungstate, and 10 cc. of 2/3N sulphuric acid. It

was found that 12 cc. of sodium tungstate and 12 cc. of sulphuric acid were necessary, with a corresponding decrease in the volume of distilled water.

(e) *Non-Protein Nitrogen.* This was estimated on the protein-free filtrate by micro-kjeldahl.

(f) *Uric Acid.* Benedict's direct colorimetric method was used (8).

(g) *Creatinine.* This was estimated by the picric acid method (9). The only difficulty encountered here was in the colour comparison; often a wide range in the depth of the unknown solution, up to 5 mm., showed apparently no change in colour when compared with the standard.

(h) *Water Content.* The moisture was estimated on 2 cc. blood, weighed and dried in vacuo over sulphuric acid for 24 hours at 39°. This was found to be sufficient time to ensure complete dryness.

All determinations of sugar, calcium, urea, non-protein nitrogen have been done in duplicate.

It is hoped that this work will be continued, to give a more complete analysis of the blood constituents, including chlorides, creatine, total acid-soluble phosphate, and iron.

HISTOLOGICAL METHODS.

Fixing and Staining of Smears.

Various reagents for fixing and staining blood smears were tried, but the following modification of Pappenheim's panoptic method (21) proved to be the most convenient and satisfactory, and was finally adopted.

Smears were dried in air, and immersed face downwards for two minutes in a Laveran-Mesnil dish containing Jenner's staining solution, immediately covered to prevent evaporation. The smears were then drained, and placed for 10 minutes in a similar dish containing Giemsa's solution diluted 10 times in boiled and cooled distilled water, after which they were washed in distilled water for 30 seconds, and dried rapidly.

Fixation was thus combined with a preliminary staining of the acidophile elements, while the deep purplish-violet colour associated with the "Romanowski" stain was not interfered with.

It is to be noted that the microscopic picture varies with the hydrogen ion concentration of the "Giemsa" diluting fluid (20); the pH should therefore be adjusted to a suitable figure before examining smears of pathological blood. This can conveniently be done by the use of buffer solutions (10).

Counting the Erythrocytes. The apparatus used consisted of a Thoma haemocytometer and a Leitz microscope with a No. 4 ocular and No. 7 objective.

Method. Owing to the difficulty of making suitable preparations for total blood counts at the abattoirs, it was found necessary to evolve a method whereby the blood sample could be transported to the laboratory in a condition suitable for examination. According to Lewis and Shope (11), potassium oxalate in the

proportion of 2 mgm. per cc. of blood prevents coagulation, and at the same time keeps the blood in a suitable state for subsequent dilution for at least 12 hours. It was found that this method was not entirely satisfactory, and after a series of tests, sodium fluoride in similar proportions was adopted as a suitable reagent. Counts were completed within 24 hours of the collection of the blood. In order to preclude the possibility of error due to settling of the corpuscles, the container was thoroughly agitated before each count. A dilution of 1 in 200 of Toisson's solution was then made in the special pipette, and the contents were thoroughly mixed by shaking. Using the ordinary precautions, the counting chamber was filled, and the corpuscles were allowed 10 minutes to settle.

Two or more counts were made from each sample, using in each case a freshly prepared dilution of blood. In cases where marked divergence occurred a large number of preparations was counted, and the final figure arrived at by taking an average of the three most nearly related totals.

Counting the Leucocytes. The apparatus was similar to that employed for counting the red cells, except that a special pipette for making a dilution of 1 in 20 was used, 0·3% acetic acid coloured with methyl violet, took the place of Toisson's solution, and No. 2 ocular served instead of No. 4 on the microscope. Blood was taken from the same samples as used for the red cell counts.

At least three, but often six, preparations from each sample were counted, refilling the pipette each time, and an average of the totals made.

Differential Counts. It was found that the smaller leucocytes generally tended to arrange themselves away from the periphery of a smear, and that a considerable number of the larger elements were frequently carried along to the end.

Two to four smears from each animal were examined, and the method of counting advised by Schilling (12) adopted, i.e., four areas were dealt with on each slide, and an equal number of cells (approximately 25–50) in each area counted.

Very little literature could be found regarding the classification of the white blood cells of cattle and sheep. However, it was finally decided to recognize the following groups:

Lymphocytes.
Large Mononuclears (Monocytes).
Polymorphonuclears.
Eosinophiles.
Basophiles.

A few cells (1–2% in apparently normal smears) did not conform with the rules laid down for membership in any of these groups, but when they appeared to belong to immature classes (i.e., myelocytes and lymphoblasts) they were treated as though they were mature cells, and placed in their corresponding groups.

As regards the recording of the counts, it was found that a sheet of paper ruled similarly to that used by Neser (13) gave excellent results, but in the later stages of this work Schilling's "Differential-Zahltafel" was employed. By either of these methods any irregularity in the distribution of the leucocytes could be immediately detected.

Cleaning of Apparatus.

Glass Slides. New slides only were used, and these were boiled in bichromate solution for half an hour, washed, drained, kept in 95% alcohol, and flamed prior to use.

Haemocytometer. After each sample of blood was dealt with the slide and cover-slips were immersed in concentrated nitric acid for 5 minutes, washed in running water, then warm water, drained, treated with alcohol, and then with ether, and allowed to dry (modification of Piney's method) (14). Between each count washing with a few drops of acid alcohol, followed by 95% alcohol, was found to be satisfactory. Pipettes were attached to a suction pump, were washed through with water, then alcohol, and lastly air.

DISCUSSION.

The complete results are given, together with the standard deviation, mean, probable error of the mean, and median for each group of figures.

The figures marked with an asterisk have been omitted from these calculations on both biological and mathematical grounds. A blood urea concentration of 103 mgm. per 100 cc. is obviously abnormal, and in such cases a *post-mortem* record would have been valuable. In the cases of high urea figures the corresponding non-protein nitrogen results are omitted, as the nitrogen, also very high, mostly consists of urea nitrogen. Low urea results, i.e., those less than 10 mgm. to 100 cc. are omitted on the grounds of hydrolysis, as the corresponding non-protein nitrogen does not give an indication of low urea nitrogen. Similarly blood sugar results less than 0·040% have been attributed to hydrolysis, as such a low concentration, if real, would have caused coma and death.

The only other results omitted are those which on drawing variant curves were seen to be well without the normal range.

We have not attempted to show any mathematical relationship between the chemical analysis and cellular contents of each sample. The discussion of results will therefore deal with the two parts separately.

1. The chemical results agree very well as a whole with those of other countries, with the notable exception of those for uric acid, which are very much higher than the maximum results found elsewhere. It is interesting to note here that a high uric acid value as compared with normals for other countries has been found in Australia for normal human beings (Green and Krieger, 15).

Further work is being done to test Benedict's theory of combined uric acid. Benedict also refers to the upsetting influence of thiasine in uric acid analyses of blood. Thiasine is a betaine-histidine compound which gives with arsenophosphotungstic acid the same colour reaction as uric acid.

The inorganic phosphate and calcium have a tendency, as would be expected, to be higher in lambs than in full-grown sheep.

No remarks need be made on the other figures relating to the chemical analysis. They agree as well as might be expected with those of other countries, and show no apparent abnormalities.

2. *Histological Results.* According to Clowson (16) the red cell counts of American cattle tend to be greater in early than in middle or late summer. The counts in this investigation were made over a period of 5 months (April-August), i.e., autumn to midwinter. The mean temperatures recorded in Melbourne during that period were: April, 59·40; May, 54·10; June, 50·40; July, 48·70, and August, 51·0. No variation according to season, however, could be noted. The cattle slaughtered were pure and crossbred, and included Jersey, Ayrshire, Shorthorn, Hereford, Red Poll, and Aberdeen Angus types. The ages varied from 2–11 years, cows averaging 7 years and bullocks 4 years. The wethers examined were of an average age of 4 years. Included under the heading "lambs" were animals from a few months to 1 year old.

Practically none of these animals could be regarded as free from parasites. Helminth infestations in varying degree are usual in the majority of sheep and cattle slaughtered at the Melbourne City Abattoirs. As well as an eosinophilia which these parasites might cause, it seems probable that local irritations and subsequent infections might account for a deviation from the normal differential count. Also the marked difference between the polymorphonuclear counts of lambs and of wethers might be associated with the known high incidence of *Caseous lymphadenitis* in older sheep in Australia. This point of disease incidence, however, has not been determined, and the difference in the counts may be normal.

As regards the actual blood used it was assumed that carotid blood would give a true indication of the elements available for distribution over the whole body, and incidentally any question of excessive local stimulation, inflammation, and destruction of tissue would not arise. The results support this view.

Da Costa (17) enunciates a number of factors influencing an erythocyte count, and no doubt these are responsible for some of the variations in our figures. Also, animals living in high altitudes have been shown to have higher red cell counts than those living in low country (16). The red cell counts obtained by us in general appear to be higher than those recorded in other countries; they also indicate that, as in the human subject, older animals have a less number of red corpuscles than young ones.

Conversely, the numbers of leucocytes present in the blood of the sheep and

cattle in this investigation are lower than the average figures noted for other parts of the world. A slight variation with age is also shown here. In addition to the greater accuracy incidental to the use of arterial blood, the period of fasting before slaughter reduces any error or fluctuation which might be due to digestive leucocytosis.

The mean differential totals in the cattle section agree very closely with statistics recently compiled in France (18), and it is interesting to note that the French workers use blood taken from the ears of 39 bovines. As investigators in other countries have shown, young animals appear to have a greater relative number of lymphocytes than older ones. Old cows were found to possess twice as many lymphocytes as polymorphonuclears, while in wethers the latter cells predominate. Cows Nos. 27, 43, 44, 62, 63, 64, certainly have departed from the probable normal, but the presence of pathological conditions is suggested by a high total leucocyte count in the case of No. 27, high ureas in Nos. 43 and 44, and an eosinophilia in No. 64.

In discussing the counting of blood cells one authority recently gave us some standards by means of which the possible ranges of error could be computed (19). If likely variations due to individual peculiarities and susceptibilities of the various animals are also considered, it will be seen that an attempt to state one set of figures as normal would be futile.

Leucocytes. As a marked similarity exists between the white cells of cattle and sheep, the chief characteristics of each class will be described, and the points of difference, if any, noted.

The Lymphocytes. These cells vary from approximately the size of a red cell to that of a monocyte. They are usually round when viewed on a smear, and consist of a nucleus, frequently eccentric in position, surrounded by a varying amount of cytoplasm.

The nucleus is usually circular in shape, but may be oval, slightly indented, kidney-shaped, lobed, rarely segmented or irregular in outline. When stained as described it has an intense purple-violet colour, which is not uniformly distributed. Deeper stained masses of nuclear material, round or irregular in shape, appear throughout the nucleoplasm, and the periphery is often well-defined by a circle of these elements. The nucleoplasm stains less intensely and rather more violet in colour, but less distinction exists between these two elements than is found in the monocyte. Rarely the more deeply stained elements radiate towards the centre, giving the nucleus the typical cart-wheel appearance.

The amount of cytoplasm varies from a bare rim in some cases to a considerable quantity in others. It is basophilic in character, and varies in intensity of colour from a light to an intense blue or blue-grey. As in the nucleus, two elements can usually be defined, a definite spongioplasm, granular in character, and staining more intensely, and a surrounding paraplasm. Generally that area

adjacent to the nucleus takes less colour than elsewhere, and a distinct perinuclear zone is found. Often in the thicker parts of a smear cells are found with cytoplasm apparently less than normal but deeper in colour. This appearance is evidently in some cases due to contraction and concentration of the cytoplasm around the nucleus.

In the large majority of smears, cells, apparently lymphocytes, are found which contain ruby-coloured granules. These cells constitute about 1–3% of the total number of lymphocytes, but in one smear, obviously from an abnormal animal, 50% of these cells contained the bodies.

The granules appear to be of two types: (1) Those which are present after a prolonged application of the Giemsa stain, and are diffuse in the cytoplasm; (2) those which are localized about one particular area. In the latter case, usually from 6–12 granules are found, generally uniform in size in any particular group, but varying from batch to batch from the size of a pinprick to that of a coccus.

The granules are frequently found opposite to an indentation in the nucleus, and, when they are present, debris like that of disintegrated platelets has sometimes been observed in the vicinity.

The large lymphocytes of sheep often seem to take the nuclear stain with less intensity than those of cattle, and the chromatin appears to be in finer masses. Up to five nucleoli-like bodies may be seen in the cells, and the cytoplasm is fairly abundant.

The Polymorpho-Neutrophiles. These cells are about 12–14 microns in size, and have the appearance of flat, circular discs when viewed in smears.

The nucleus is usually quite irregular in shape, appearing as a ribbon somewhat convoluted, or as a number of lobes joined together by fine cotton-like strands. Sometimes as many as eight lobes are found, but 1, 2, or 3 are the usual numbers. The stain used gives the nuclear material a deep purple appearance, somewhat similar to that of the lymphocyte. Deeply stained bodies are found in the nucleus, but the nucleoplasm is often a little lighter than that of the lymphocyte.

The cytoplasm is scarcely visible, and is packed with minute faint pink refractile granules. Rarely and probably in pathological cases larger ruby-like points may be seen.

In addition, intermediate stages between the myelocyte and the mature polymorph are frequently seen. Sometimes the number of band forms reaches 3–4% of the total white cells, when an occasional metamyelocyte may be found. Also a form resembling a polymorph, except that it has up to four large lobes almost filling the cell, may, but rarely, occur. The lobes appear like flattened nuclei. Rarely the cytoplasm appears faintly violet instead of oxyphile.

Large Mononuclears. Considerable confusion seems to exist regarding the classification of these cells, which are the largest elements found in normal blood.

They range from approximately the size of a large lymphocyte to about 20 microns, and a number of distinct types appear to exist.

The nucleus is large, usually eccentric, and varies considerably in conformation. In some types it is round, but more often it assumes any intermediate form between round and extremely irregular. Horseshoe shapes and three-lobed forms frequently occur. The nucleus of the typical monocyte takes the purple-violet stain less intensely than the lymphocyte. The chromatin appears in the form of threads, and interwoven among the threads are spindle-shaped or scale-like, deeply-stained masses. Three or four circles, marked off by deep purple circumferences, are often present throughout the nucleus. The nucleoplasm is lighter in colour than that of the lymphocyte, and the peripheral area of the nucleus is not so definitely defined.

The amount of cytoplasm is usually abundant. It has a definite affinity for the basic stain, varying in colour from a faint blue to a deep blue-grey. In the former case a well-defined border is frequently found. The cytoplasmic material certainly does not appear to be homogeneous in nature. Sometimes one finds the ground-glass appearance, but more often a spongioplasm is seen, which stains uniformly (or even with increasing intensity) from outer edge to nucleus. If overstained with Giemsa solution small ruby granules may appear in the cytoplasm.

At least three types of these cells may therefore be found: (1) The large so-called hyaline cell; (2) the smaller cell, with the protoplasm stained a deeper colour, and having a distinct granular reticulum; (3) the old "transitional" type described by Ehrlich.

Eosinophiles. Eosinophiles are a little larger than polymorphs, but have a similar shape.

In appearance the nucleus is most irregular, and may take the form of a ribbon-like band, or be lobed, or even segmented. In the last case the strands of material can sometimes be found connecting the divisions, but often the two or three segments are apparently isolated. In structure these nuclear lobes are not unlike those of the polymorphonuclear, but the chromatin material does not stain with the same intensity.

The cytoplasm appears to be basophilic, but is packed with round granules, staining a brick-red colour with eosin, which often appear to be laid down along definite lines, and not haphazardly. These granules are not dissimilar in cattle and sheep, but are distinctly smaller than those found in the horse.

Basophiles. These cells are only occasionally seen in blood smears, but two types have been noted: (1) about the size of, or a little larger than, an eosinophile, and containing large, intense purple, bead-like granules; (2) about the size of a neutrophile, but having smaller granules of a similar character, but not uniform in size. The nucleus is not as easily defined as in other types of cells, but stains intensely with the basic stain, and sometimes several segments are discernible.

Atypical Leucocytes. (a) In both cattle and sheep a cell is found which appears to be intermediate in nature between the typical lymphocyte and the monocyte. It is usually approximately the size of a large lymphocyte, and has a varying amount of cytoplasm.

The nucleus is often in the centre of the cell, but may be pushed to one side, especially when ruby granules are present in an area of the cytoplasm. It takes the stain with less intensity than the nucleus of the lymphocyte, and does not contain the deep masses of chromatin which are characteristic of that class. The clear differentiation between the two elements in the nucleus, as seen in the typical monocyte, is not apparent, and yet the structure is of the fine fibrillar type, with elongated masses, and sometimes several coarser clumps of chromatin. A definite deeper-stained area can frequently be seen towards the edge, and the nucleus itself is surrounded by a lightly coloured zone.

The cytoplasm usually has the granular, reticulated appearance, spongioplasm appearing grey-blue in colour, and the paraplasm faintly basophilic. Granules of the type found in smaller cells may be present, and their presence often suggests a phagocytic origin.

(b) Another type of cell frequently found, especially in young sheep, comprising up to 4% of the leucocytes, is nearly as large as a large lymphocyte (but is sometimes found up to 15 mic. in size). The nucleus stains with the intensity peculiar to that cell, but instead of the usually large and deeply stained clumps of chromatin, diffuse granules, often arranged in a reticular form, are found.

The cytoplasm is not very abundant, and contains a deep blue, granular spongioplasm.

This cell is sometimes seen in smears of cattle blood. The nucleus may be irregular in shape, and stains a little lighter than that of a lymphocyte.

(c) Frequently cells appear which are from 12–14 microns in size, but are atypical of any group. The nuclei stain a purple-violet colour, lighter than that of a lymphocyte, and are either granular or contain spindle-shaped pieces of chromatin. Nucleoli-like bodies (often four) and circular areas, defined by intensely coloured peripheries, are seen.

The cytoplasm is usually scanty, and may be intense blue or light grey-blue in colour, with well-marked structure. A series of vacuole-like areas in the cytoplasm appear to surround the nucleus, and sometimes coccus-like bodies are present.

In addition, cells resembling the Rieder and Plasma forms, and others of extremely doubtful classification, appear occasionally.

SUMMARY.

The blood of sheep and cattle has been quantitatively examined for the following constituents: Sugar, calcium, urea, non-protein nitrogen, uric acid, creatinine, inorganic phosphate, and moisture.

The total red and white cell counts, with the differential count, have also been made.

The results are given in tabular form, together with the mean, standard deviation, probable error of the mean, and median of each group of figures. A description of the leucocytes found in the animals is also given.

In conclusion, the authors wish to express their sincere thanks to Associate Professor W. J. Young, in whose department the biochemical work was carried out, and to Mr. H. E. Albiston, Acting Director of the University Veterinary Research Institute, in whose department the histological work was carried out. We also wish to thank Mr. Belz for his assistance in the statistical results; Professor Woodruff and Mr. A. Turner for their interest and valued criticism. Finally we wish to acknowledge our indebtedness to the Superintendent and staff of the City Abattoirs, Melbourne, for their assistance throughout the investigation.

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TABLE 1.

Particulars of the Animals.	Sugar per cent.	Calcium mgm./100 c.c.	Urea mgm./100 c.c.	Urea N. mgm./100 c.c.	N.P.N. mgm./100 c.c.	Uric Acid mgm./100 c.c.	Creatinine mgm./100 c.c.	Inorg. Phos. mgm./100 c.c.	Moisture per cent.	Sample No. for reference.	Erythrocytes per cmm.	Leucocytes per cmm.	Lymphocytes. %	Monocytes. %	Polymorphonuclear %	Eosinophiles. %	Basophiles. %	Unclassified.
Sheep	0·071	*13·34	38·90	18·14	32·64	3·09	1·00	—	—	1								
	0·049	*14·42	27·50	12·83	20·90	2·93	0·83	—	—	2								
	*0·038	*14·10	14·50	6·76	21·26	2·13	—	—	—	3								
	0·056	*13·70	*63·30	*24·86	—	3·08	0·73	—	—	4								
	0·063	*12·72	23·65	11·03	25·56	2·59	0·72	—	—	5								
	*0·028	*13·92	14·41	6·72	20·32	2·79	0·75	—	—	6								
	0·079	9·76	30·80	14·37	—	3·64	1·20	5·00	80·55	7								
	0·054	10·22	25·63	11·95	—	3·92	1·16	6·35	78·04	8								
	0·060	11·12	*51·15	*23·86	—	3·70	1·22	7·48	82·01	9								
	0·065	9·66	*50·60	*23·60	—	3·15	1·30	7·14	—	10								
	0·063	—	29·15	13·60	—	—	1·61	4·06	78·94	11								
	0·062	—	22·00	10·26	—	—	1·32	5·30	80·55	12								
	*0·039	—	35·70	16·65	—	—	1·21	5·00	78·36	13								
	0·068	—	*50·05	*23·34	—	—	1·43	4·88	80·65	14								
	0·101	8·72	*103·18	*48·17	*62·52	3·61	1·77	6·56	—	64	13,100,000	4,000	23	14	63	—	—	—
Wethers	0·096	10·12	13·42	6·26	32·65	4·23	1·09	4·47	—	19	*16,100,000	3,100	38	1	50	—	1	
	0·060	10·78	28·60	13·34	37·27	3·42	1·33	4·70	—	20	*16,400,000	7,400	—	—	—	—	—	
	0·054	9·48	23·87	11·13	21·87	4·30	1·63	5·76	81·43	27	10,400,000	3,700	38	4	48	*10	—	
	*0·035	8·74	23·87	11·13	20·64	4·12	1·50	6·45	82·54	28	12,000,000	5,900	32	11	39	*17	1	
	0·075	9·72	26·29	12·26	23·10	4·42	1·45	4·70	79·67	31	10,000,000	6,100	—	—	—	—	—	
	0·107	9·30	*58·08	*27·09	*40·66	4·62	1·43	4·88	80·07	32	12,400,000	7,100	37	7	52	4	—	
	0·094	10·52	25·74	12·01	26·18	3·74	1·35	6·02	79·54	39	*14,100,000	4,900	39	8	53	—	—	
	0·065	9·80	25·85	12·06	30·42	3·39	1·34	6·06	78·68	40	13,300,000	6,200	40	3	54	3	—	
	0·058	9·62	19·80	9·24	33·26	3·88	1·36	5·03	78·68	41	13,100,000	6,000	48	5	43	4	—	
	0·079	11·12	*7·59	*3·54	28·95	3·94	1·41	5·23	—	43	—	—	48	5	47	—	—	
	0·089	9·66	17·27	8·06	29·88	3·64	1·33	6·02	—	44	—	—	33	5	62	—	—	
	0·118	10·34	44·77	20·90	27·41	3·40	1·25	5·03	—	45	*15,500,000	4,000	31	6	62	—	1	
	0·115	8·92	38·72	18·07	28·95	3·92	1·42	6·25	—	46	11,800,000	4,500	*23	6	*71	—	—	
	*0·038	9·88	46·75	21·82	40·66	3·80	2·14	6·96	79·16	47	12,500,000	5,100	47	5	48	—	—	
	0·069	9·70	26·07	12·16	32·96	4·50	2·00	6·65	78·55	48	12,900,000	5,700	31	9	60	—	—	
	0·065	9·38	33·55	15·66	29·88	4·68	2·00	6·40	79·26	49	9,700,000	5,900	37	2	60	1	—	
	0·093	10·92	*62·92	*29·37	*51·41	4·28	2·07	4·60	78·82	50	11,700,000	*8,200	35	4	60	1	—	
	0·096	8·62	35·86	16·74	32·04	5·16	1·45	5·56	—	51	11,100,000	6,600	46	10	40	1	—	3
	0·111	9·74	18·04	8·43	19·25	3·35	1·26	5·06	—	52	10,600,000	6,800	43	3	54	—	—	
	0·084	9·46	16·50	7·70	25·87	4·57	1·40	4·21	—	53	11,000,000	6,200	40	7	51	1	—	
	0·80	9·46	32·89	15·35	39·12	4·44	1·36	5·80	—	54	11,900,000	4,200	*51	*21	*27	1	—	1
	0·087	10·78	*80·41	*37·54	*62·22	4·10	1·20	5·23	—	56	9,600,000	3,800	*51	*20	*28	1	—	
	0·099	11·12	24·09	11·25	23·72	3·80	1·46	5·33	—	63	12,900,000	3,000	37	12	49	2	—	
Mean	0·078	9·88	20·14		28·19	3·77	1·26	5·57	79·75		11,600,000	5,300	39	6	52	1		
Standard Deviation	0·025	0·733	19·99		5·91	0·673	0·620	0·853	1·261		1,000,000	400	5		5			
Probable Error	±0·0027	±0·09	±0·250		±0·797	±0·078	±0·069	±0·102	±0·201		±200,000	±100	±1		±1			
Median	0·075	9·72	25·85		28·95	3·80	1·35	5·30	79·26		11,800,000	5,700	38		51			

TABLE 2.

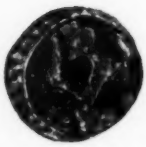
Particulars of the Animals.	Sugar per cent.	Calcium mgm/100 c.c.	Urea mgm/100 c.c.	Urea N. mgm/100 c.c.	N.P.N. mgm/100 c.c.	Uric Acid mgm/100 c.c.	Creatinine mgm/100 c.c.	Inorg. Phos. mgm/100 c.c.	Moisture per cent.	Sample No. for reference.	Erythrocytes per cmm.	Leucocytes per cmm.	Lymphocytes.	Monocytes.	Polymorphonuclear	Eosinophiles.	Basophiles.	Unclassified.
													%	%	%	%	%	
Lambs	0·058	10·90	19·58	9·13	—	2·72	1·74	5·43	80·69	15	14,900,000	7,000	56	1	40	1	—	2
	0·062	11·32	21·12	9·85	—	2·88	1·20	5·44	80·98	16	11,400,000	6,400	46	—	54	—	—	—
	0·054	10·52	38·50	17·96	29·87	2·69	1·43	5·16	80·09	17	14,600,000	4,800	66	—	34	—	—	—
	0·053	11·92	29·04	13·54	33·27	2·69	1·33	5·88	78·57	18	*20,000,000	5,600	72	1	26	1	—	—
	0·060	9·78	13·97	6·52	31·72	3·52	1·21	5·71	—	21	13,700,000	7,100	—	—	—	—	—	—
	0·055	9·42	18·70	8·72	33·88	3·43	1·27	5·71	—	22	13,400,000	3,800	—	—	—	—	—	—
	0·046	11·50	19·69	9·18	16·32	4·44	1·36	6·50	81·11	23	13,700,000	7,400	57	—	42	1	—	—
	0·054	11·20	39·60	18·47	32·03	4·44	1·29	*10·81	80·26	24	14,000,000	4,200	62	8	28	—	—	2
	0·044	9·70	23·21	10·83	33·57	4·60	1·27	9·41	—	25	13,700,000	4,200	50	5	45	—	—	—
	0·072	10·06	18·04	8·41	27·10	4·02	1·46	8·16	81·42	26	12,900,000	5,800	66	3	27	2	—	2
	0·045	10·12	39·05	18·21	38·19	4·02	1·51	6·72	80·98	29	11,700,000	2,900	54	9	36	1	—	—
	0·058	9·84	14·74	6·88	25·26	4·23	1·23	6·35	80·04	30	14,700,000	7,800	52	8	39	1	—	—
	0·087	8·68	30·80	14·37	29·57	4·40	1·70	4·97	80·35	33	10,300,000	4,200	53	5	41	1	—	—
	0·085	9·74	36·30	16·93	34·19	4·68	1·45	4·85	80·20	34	12,600,000	6,900	41	11	40	7	1	—
	0·069	11·52	15·73	7·34	26·18	3·70	1·23	5·06	79·96	35	13,100,000	3,900	70	15	13	2	—	—
	0·076	10·12	32·89	15·34	29·88	3·74	1·17	5·52	79·93	36	11,900,000	6,000	50	8	41	1	—	—
	0·069	10·20	41·14	19·19	32·96	3·83	1·17	6·20	80·09	37	13,600,000	4,300	51	16	30	3	—	—
	0·058	11·92	28·27	13·19	25·87	3·59	1·28	4·50	79·45	38	14,500,000	5,600	52	9	39	—	—	—
	0·080	10·12	38·17	17·82	28·03	3·20	1·38	6·25	—	42	10,600,000	5,500	68	7	25	—	—	—
	0·067	11·81	36·97	17·26	47·74	4·12	1·30	5·06	—	55	13,000,000	3,600	55	12	31	2	—	—
	0·072	10·78	—	—	32·96	3·92	1·20	—	—	57	12,000,000	6,600	33	32	32	3	—	—
	—	11·58	30·03	14·02	31·72	3·64	0·97	4·70	—	58	12,400,000	8,000	40	27	30	2	—	1
	0·075	10·30	22·33	10·43	14·48	3·51	1·25	8·89	—	59	13,800,000	*9,300	62	12	24	1	—	1
	0·064	10·10	*9·35	*4·63	15·09	3·83	1·38	6·96	—	60	*15,400,000	5,300	52	6	40	1	—	1
	0·042	9·52	36·41	17·00	27·10	4·17	1·27	9·09	—	61	12,800,000	4,200	65	18	16	1	—	—
	—	9·60	31·90	14·89	26·18	3·51	1·25	8·08	—	62	12,900,000	6,900	46	14	40	—	—	—
	0·078	11·18	22·00	10·27	24·33	3·76	1·00	7·62	—	65	13,200,000	5,100	67	5	26	2	—	—
	0·061	12·08	26·07	12·16	22·48	3·51	1·30	5·78	—	66	*16,900,000	4,800	*30	11	*59	2	—	—
Mean	0·063	10·55	27·85		28·84	3·74	1·31	6·31	80·27		13,000,000	5,500	55	9	34	1		
Standard Deviation	0·014	0·896	8·581		6·999	0·595	0·138	0·607	1·106		1,300,000	1,300	12		7			
Probable Error	±0·0019	±0·114	±1·135		±0·926	±0·076	±0·018	±0·079	±0·193		±200,000	±200	±2		±1			
Median	0·061	10·20	26·07		29·57	3·74	1·27	5·78	80·20		13,100,000	5,500	54		34			

TABLE 3.

Particulars of the Animals.	Sugar per cent.	Calcium mgm/100 c.c.	Urea mgm/100 c.c.	Urea N. mgm/100 c.c.	N.P.N. mgm/100 c.c.	Uric Acid mgm/100 c.c.	Creatinine mgm/100 c.c.	Inorg. Phos. mgm/100 c.c.	Moisture per cent.	Sample No. for reference.	Erythrocytes per cmm.	Leucocytes per cmm.	Lymphocytes.	Monocytes.	Polymorphonuclear	Eosinophiles.	Basophiles.	Unclassified.
													%	%	%	%	%	
Calves and Heifers	*0·149	8·12	14·96	6·98	20·32	4·73	1·22	4·57	78·17	17	17,500,000	6,300	62	—	38	1	—	—
	0·103	10·12	7·92	3·73	21·87	5·48	1·33	4·73	79·32	19	14,700,000	9,800	67	6	20	6	1	—
	0·101	12·12	—	—	20·33	5·23	1·22	3·30	77·45	20	9,200,000	5,400	75	1	23	1	—	—
	0·111	11·00	23·10	10·77	27·72	5·71	1·20	5·63	76·86	28	13,800,000	10,200	70	13	17	—	—	—
	0·095	11·62	21·12	9·86	16·32	5·63	1·35	3·29	78·53	40	9,400,000	4,100	69	8	18	4	—	1
General, Cattle	0·056	13·90	20·90	9·75	—	4·60	2·22	—	—	1								
	0·093	13·70	15·40	7·19	—	4·49	2·17	—	—	2								
	0·109	12·10	21·67	10·11	—	4·52	1·96	—	—	3								
	—	13·80	9·35	4·36	—	4·60	2·33	—	—	4								
	—	13·90	12·65	5·90	—	4·71	2·07	—	—	5								
	—	13·90	9·46	4·41	—	4·71	1·88	—	—	6								
	*0·131	13·12	31·35	14·63	—	5·00	1·56	5·71	78·83	9								
	0·113	14·06	25·52	11·91	—	4·50	1·27	5·71	80·08	10								
	0·084	11·32	8·14	3·80	—	4·37	1·23	4·06	80·34	11								
	0·081	13·32	11·00	5·13	—	4·59	1·44	5·06	79·01	12								
	0·069	12·52	17·60	8·21	—	4·76	1·31	5·10	79·30	13								
Bullocks	0·087	10·12	19·47	9·08	26·48	5·06	1·42	4·85	78·10	15	11,500,000	4,600	*39	—	*42	*18	—	1
	0·084	10·40	18·70	8·72	24·94	4·94	1·22	4·08	75·37	16	10,600,000	4,600	68	2	23	5	—	2
	0·082	9·18	11·33	5·28	25·87	4·88	1·34	4·27	77·01	18	8,500,000	5,300	75	1	21	3	—	—
	*0·129	10·74	22·00	10·26	23·15	5·76	1·52	4·00	78·94	21	11,600,000	5,000	70	4	21	5	—	—
	0·090	9·52	25·19	11·75	23·10	5·33	1·32	4·35	76·75	22	11,800,000	4,300	67	4	20	7	1	1
	0·069	10·72	30·80	14·37	26·49	5·88	1·73	3·90	76·65	23	10,400,000	5,800	61	4	20	*15	—	—
	0·073	12·38	28·60	13·34	38·81	5·71	2·31	3·57	76·06	24	10,600,000	6,800	56	6	32	4	—	—
	0·100	10·58	10·45	4·87	34·80	5·80	2·26	6·15	76·50	25	11,200,000	5,000	70	4·5	15	*10·5	—	2
	0·112	9·02	10·78	5·03	34·19	5·52	1·94	5·84	76·79	26	10,300,000	7,800	79	4	13	4	—	—
	0·090	10·20	13·20	6·16	22·48	*7·62	1·58	5·03	77·62	29	10,700,000	5,700	60	13	20	7	—	—
	0·074	10·38	18·70	8·72	23·72	3·96	1·00	5·16	76·25	30	10,300,000	4,100	67	12	15	6	—	—
	0·107	11·30	15·62	7·29	19·09	6·35	1·33	5·00	76·95	32	10,600,000	6,400	—	—	—	—	—	—
	*0·129	11·92	16·05	7·49	17·86	*7·27	1·06	4·79	75·81	33	9,000,000	3,000	—	—	—	—	—	—
	0·108	9·72	17·38	8·11	22·48	4·44	1·46	3·92	77·03	35	9,100,000	4,900	67	12	15	6	—	—
	0·108	10·80	25·19	11·75	26·80	4·30	1·96	4·10	76·22	36	*7,500,000	6,200	48	5	47	—	—	—
	*0·124	10·14	21·34	9·95	27·10	3·86	1·41	5·88	77·38	37	8,500,000	5,900	50	5	40	5	—	—
	0·079	12·46	31·63	14·76	17·87	6·20	1·42	4·26	79·61	46	*6,800,000	7,400	*36	9	*51	4	—	—
	0·094	11·12	15·95	7·45	44·04	5·10	1·40	3·33	—	57	9,900,000	6,100	47	9	41	3	—	—
	0·086	10·24	23·10	10·78	23·10	4·88	1·69	5·40	—	59	10,600,000	3,900	59	9	26·5	5·5	—	—
	0·104	11·02	7·70	3·59	14·48	4·97	1·28	5·10	—	65	9,200,000	4,200	64	8	20	8	—	—
	0·086	10·28	17·16	8·01	20·33	5·16	1·40	3·94	—	66	10,400,000	7,600	51	9	36	4	—	—
Mean	0·091	11·37	18·34		24·76	5·02	1·56	4·65			10,300,000	5,500	62	6	25	5		
Standard Deviation	0·0164	1·584	6·009		6·721	0·605	0·381	0·79			900,000	1,000	13		10			
Probable Error	±0·0021	±0·176	±1·486		±1·954	±0·069	±0·042	±0·09			±100,000	±100	±2		±2			
Median	0·090	11·00	17·38		23·10	4·94	1·42	4·73			10,400,000	5,300	64		21			

TABLE 4.

Particulars of the Animals.	Sugar per cent.	Calcium mgm/100 c.c.	Urea mgm/100 c.c.	Urea N. mgm/100 c.c.	N.P.N. mgm/100 c.c.	Uric Acid mgm/100 c.c.	Creatinine mgm/100 c.c.	Inorg. Phos. mgm/100 c.c.	Moisture per cent.	Sample No. for reference.	Erythrocytes per cmm.	Leucocytes per cmm.	Lymphocytes. %	Monocytes. %	Polymorphonuclear %	Eosinophiles. %	Basophiles. %	Unclassified.
Cows	0·077	*12·72	23·10	10·78	21·56	5·23	1·37	4·23	80·95	7								
	0·094	*12·92	22·44	10·47	—	4·04	1·25	2·90	79·10	8								
	0·066	10·72	14·30	6·67	34·80	4·65	1·27	3·67	81·89	14	7,000,000	6,300	—	—	—	—	—	—
	0·078	9·72	28·27	13·19	27·41	6·96	1·39	6·45	77·49	27	8,300,000	*8,200	*27	15	50	8	—	—
	0·073	9·78	29·04	13·55	21·87	6·67	1·50	5·33	78·48	31	6,800,000	3,300	—	—	—	—	—	—
	0·060	10·12	23·65	11·03	24·95	6·50	1·38	4·21	74·74	34	*10,500,000	6,200	—	—	—	—	—	—
	0·098	11·16	11·22	5·23	14·48	3·69	1·47	4·76	79·54	38	8,300,000	4,300	67	8	20	5	—	—
	0·088	9·52	20·68	9·65	22·65	5·63	1·53	5·40	78·94	39	8,000,000	4,300	53	7	26	13	—	1
	0·101	11·72	10·45	4·88	14·78	5·76	1·72	4·44	76·74	41	8,900,000	5,200	46	13	24	16	—	1
	*0·124	9·92	35·75	16·69	33·27	5·44	1·84	6·67	75·42	42	*10,300,000	4,800	42	12	35	9	—	2
	0·105	10·52	*46·86	*21·87	28·64	6·02	1·54	3·64	78·42	43	7,400,000	4,200	*29	13	*50	8	—	—
	*0·134	10·28	*48·57	*22·67	29·88	5·67	1·50	2·50	76·72	44	8,400,000	6,600	*29	4	*66	1	—	—
	0·089	10·48	32·01	14·95	24·02	5·84	1·26	4·23	76·62	45	7,300,000	*8,200	69	3	25	3	—	—
	*0·120	10·52	11·99	5·60	—	6·35	1·64	4·52	—	47	8,600,000	*8,400	42	8	41	7	1	1
	*0·159	*12·48	12·65	5·90	—	5·63	1·46	5·48	—	48	8,000,000	6,200	48	10	41	1	—	—
	0·072	10·52	15·18	7·09	—	6·15	1·50	5·03	—	49	9,100,000	4,800	63	7	17	12	—	1
	0·085	10·92	17·38	8·11	—	6·02	1·17	4·76	—	50	6,700,000	4,000	70	8	15	7	—	—
	0·087	9·38	22·22	10·32	—	6·02	1·22	4·85	—	51	7,300,000	3,800	48	11	31	10	—	—
	0·070	9·48	32·67	15·26	—	5·97	1·46	2·92	—	52	7,800,000	4,800	41	13	41	4	1	—
	0·081	10·52	23·54	10·99	—	6·84	1·47	4·62	—	53	9,400,000	3,200	51	11	30	7	—	1
	0·086	11·24	22·55	10·53	26·79	6·15	1·36	3·65	—	54	6,900,000	4,600	57	10	24	9	—	—
	0·090	11·10	18·81	8·78	29·88	5·26	1·53	5·56	—	55	8,400,000	5,900	56	14	15	15	—	—
	0·103	11·02	8·80	4·11	17·25	5·16	1·36	2·98	—	56	9,400,000	6,900	43	12	27·5	17·5	—	—
	0·100	11·44	29·26	13·66	36·34	5·56	1·30	3·21	—	58	7,200,000	3,900	60·5	8	23·5	7·5	0·5	—
	0·049	11·62	34·54	16·12	25·87	5·10	1·83	5·84	—	60	7,900,000	5,900	63	12	21	2·5	0·5	1
	0·050	9·78	19·25	8·98	18·33	4·73	1·76	4·17	—	61	7,000,000	6,400	52·5	6·5	29·5	11·5	—	—
	0·046	11·12	17·82	8·32	21·25	5·06	1·38	4·70	—	62	7,600,000	3,500	*33·5	7	*52	7·5	—	—
	0·087	10·52	10·34	4·83	17·56	5·23	1·30	3·83	—	63	6,900,000	4,400	*28	6	*58	8	—	—
	0·095	11·02	19·47	9·09	17·86	4·94	1·43	3·46	—	64	6,900,000	3,700	*25	2	*39	*33	1	—
Mean	0·081	10·54	21·01		24·26	5·60	1·46	4·44	78·08		7,800,000	4,900	54	9	27	8		
Standard Deviation	0·076	0·723	7·711		6·246	0·722	0·119	1·057	2·016		900,000	1,000	9		8			
Probable Error	±0·0024	±0·096	±1·002		±0·919	±0·090	±0·015	±0·132	±0·377		±100,000	±100	±1		±1			
Median	0·086	10·52	20·68		24·02	5·63	1·46	4·41	78·42		7,800,000	4,800	53		25			



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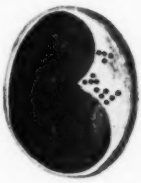

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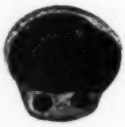

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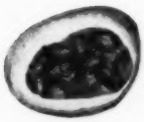

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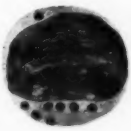
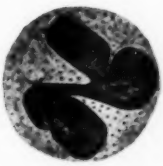


1f

2a



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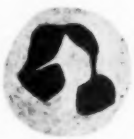

3a

3b

4

5

DESCRIPTION OF PLATE.

1. **Lymphocytes.**
   ('a', 'b', and 'c' being of greater magnification than 'd', 'e', 'f'.)
   'b', 'd', 'f' illustrate types of granules found in these cells.
2. **Monocytes.**
   'b' shows the effect of prolonged staining with 'Giemsa'.
3. **Polymorphonuclears.**
   (a) Mature.
   (b) Band form.
4. **Eosinophile.**
5. **Type of Basophile.**

DERMATOMYCOSIS OF THE SHEEP (LUMPY OR MATTED WOOL) DUE TO *ACTINOMYCES DERMATONOMUS (n.sp.)*

by

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INTRODUCTION.

The present paper describes the results of an investigation into the cause of a skin disease of sheep in South Australia. A preliminary communication was made earlier in the year (2). When this investigation was started in September, 1928, there was nothing in the literature to indicate that the disease had been described from any part of the world. Since then Bekker (1) has described, under the name "lumpy wool," a condition observed quite commonly in South Africa in many parts of the Union. It makes its appearance sporadically in merino sheep. Hard pyramidal masses, formed by an exudate into the wool, are found on the dorsal aspect of the body. Attempts to establish the cause of the condition and to reproduce it artificially have failed. It is regarded as of considerable economic importance, as much wool may be damaged, and in some cases the general health of the animal be impaired. It is possible that the condition described by Bekker has the same etiology as that found in this State. Seddon (3) has described a similar condition in sheep in New South Wales. It was found in several widely separated localities. Seddon has succeeded in conveying the disease to healthy sheep under experimental conditions. An opportunity occurred in May last of seeing some of these experimental animals and some of the specimens collected in the field. The opinion was reached that the condition under investigation in New South Wales is probably identical with that observed in this State.

From time to time specimens of matted wool have been submitted for an opinion as to the probable cause of the condition. No definite information was obtained from macroscopic or microscopic examinations of these specimens. Later, opportunity occurred of examining in the field animals showing a matted

condition of the wool and of keeping some affected animals under observation in the laboratory. Experimental and bacteriological examinations have been conducted, and the results of these examinations are given below.

Clinical and Field Observations.

Most of the affected animals examined have shown numerous small areas of densely matted wool scattered irregularly over the back, the sides, and the upper-surface of the neck. Usually the ears, face, legs, and under-surface of the body are quite free from any lesions. In a few instances small lesions have been observed about the nose and lips. In one exceptional case (sheep L. 1) there were extensive lesions about the face and ears, as well as on other parts of the body. This animal was kept under observation at the laboratory for some time, and a more detailed description of the lesions in this case will be given below.

The matted wool is attached very firmly to the skin, the areas affected being roughly circular, and varying in diameter from slightly less than a quarter of an inch to about 1 inch. For the most part these areas are discrete, but sometimes two or more may be situated closely together and coalesce. The matted condition of the wool extends up along the fibre for about an inch to an inch and a half. In exceptional cases the matted condition may extend throughout the whole length of the staple. These masses cannot be removed from the skin except by cutting with scissors or other sharp instruments, when some damage to the skin results, particularly around the periphery, where adhesion is greatest. The skin beneath is found to be raw-looking. The mass of matted wool is found to be concave on its proximal surface, due to contraction of the centre, and the binding together of the wool fibres is found to have resulted from an exudation from the skin which has dried into a dense, tough mass. In exceptional cases the mass may be removed from the skin by moderate traction, leaving a dry but otherwise normal-looking skin beneath.

Only young animals have been found to be affected. The condition has been observed at shearing time, when it has been found impossible to pass the shears through the matted areas. On one property, M.L., summer lambs, about seven or eight months old, were found most severely affected, while the previous season's lambs, sheep of about eighteen months old, were less severely affected. Older sheep, even when running with the younger, affected sheep, have not shown the presence of any lesions. Sixteen out of 60 summer ewe lambs (26%) and 22 out of 200 eighteen months old ewes (11%), were found to be affected. On another property, L., about 10% of the young ewes were affected.

Field observations were confined to the sheep on these two properties in the same district, about 130 miles north of Adelaide, and separated from one another by a distance of about 3 or 4 miles. The two flocks were quite distinct, and there had never been any contact between them. Both flocks were stud merinos,

one a fine wool type and the other a stronger wool type. In the one instance only an odd animal had been affected during the previous four years, and in the other one ewe was found with the disease three years previously, but it had been destroyed. From the depth of the exudate in the wool one judges that the condition had started about a month or so before shearing, that is, during June or July, and when the animals were seen in the latter part of September there was distinct evidence that the lesions were clearing up in the majority of the animals, normal, clean wool pushing the matted wool away from the skin.

One animal of the stronger wool type and two from the other stud were kept under observation at the laboratory. One of the animals (sheep L. 1) from the latter stud showed the disease in an advanced and severe form. The exudate extended throughout the whole length of the staple, showing that the condition had progressed during the major portion of the year. The animal was two and a half years old, the only animal over eighteen months old found affected in either stud. The skin and wool on the back of the animals was very extensively involved, some of the areas being 4 or 5 inches in diameter. Wart-like masses were crowded together over the nose, and extended downwards to the corners of the mouth and under the chin (*vide* figure 1). The external ears were also affected and eroded along the margins. Some of the matted wool could be removed easily from the skin, leaving a dry but otherwise normal-looking skin beneath. The animal was kept under cover, but the condition was progressive. Large masses of matted wool came away from the lumbar region, leaving a thick, dry, rough, and scaly mass attached to the skin. Lesions developed on the legs as far as the hoofs, and those on other parts of the body enlarged. There was, however, no involvement of the ventral surface. The animal became emaciated, and died towards the end of January.

The other animal from the same stud, a ewe lamb (sheep L. 2), has also shown a progressive condition, with an exudate through the whole length of the staple at the time of writing, after being under cover and under observation for twelve months. Lesions have also appeared about the upper lips (*vide* figure 2). The lesions about the trunk have not increased very much in diameter, although the exudate has been continuous, and, as far as can be judged, new lesions have not developed about the trunk. The dorsal surface is comparatively free from lesions, most of them being situated along the sides of the thorax and the flanks. The ventral surface remains free from any lesions.

The stud M.L. was transferred to another State, and no information has been received as to the health of the animals.

There has been little recurrence of the disease this year in Stud L. No prophylactic measures have been adopted. Three thousand sheep have been shorn, and only six very slightly affected animals have been detected. Possibly the droughty conditions were responsible for the low morbidity. The rainfall

in the district in the years 1928 and 1929 over a period of four months is set out below:

	1928.	1929.
May	208 points	139 points
June	258 „	162 „
July	216 „	117 „
August	49 „	132 „

It will be seen that 682 points fell in the May–July period in 1928, and 418 points during the same period in 1929. It is possible that the difference of 264 points was responsible to some extent for the low morbidity.

Experimental Transmission.

The first attempt to reproduce a lesion in a healthy animal was made by removing a mass of matted wool from the skin of an affected sheep and rubbing the base of this mass over a small area of skin on the nose of a healthy three years old merino ram. The area inoculated was lightly rubbed with a scalpel until a slight exudate appeared, after which the base of the mass of matted wool was rubbed over the area. On the following days an increasing swelling of the skin, with the accumulation of a dried exudate, developed. The reaction produced was only transitory. A scab about a quarter of an inch thick developed, but was pushed off by the growth of wool in about three weeks' time.

Two other areas of skin in the same animal were inoculated, one on the forehead, where the wool had been clipped with scissors, and the other on the sides of the thorax, where the wool was merely parted. In neither instance was the skin scarified. The same transient reaction occurred, with matting together of the wool by exudate.

These experiments were repeated on the same ram, and another ram of the same age, using material from affected lambs from both the studs mentioned. In every case the same type of reaction was produced following the application of the base of a freshly removed mass of matted wool to the uninjured skin.

Two merino lambs were used for the same type of experiment, the wool being parted and the skin rubbed with the base of a freshly removed mass of matted wool from an affected animal. In no case was the rubbing severe enough to produce any macroscopic damage to the skin of the experimental animal. In from 48 to 72 hours an exudate had occurred into the wool covering the part inoculated. The exudate continued to increase for a week or ten days, matting the wool together to a depth of about a quarter of an inch or slightly more. The reaction ceased after a period of from ten days to a fortnight, and with the growth of the wool the matted areas were pushed off the skin. The reaction produced was somewhat more severe than that produced in the older animals, but not as severe as that found under natural conditions.

Microscopic examinations of the matted wool had shown the presence of numerous squamous epithelial cells and leucocytes, as well as a short, branching

mycelium. The mould-like micro-organism had been isolated, and pure cultures were used in transmission experiments.

An experimental lamb (merino) was sprayed with water from a hose until the fleece was soaked, on two consecutive days. On the third day three lesions were removed from an affected lamb, and the base of each ground up in a mortar with sterile saline. This emulsion and a culture of the micro-organism, consisting of a mixture of a 24 hours and a 48 hours old culture in glucose broth were taken for the purpose of inoculation. In one area the culture was applied to the skin after parting the fleece. In another area the culture was applied to the wool about one-half to three-quarters of an inch from the skin. The culture was also applied to the skin of the groin after it had been cleaned with alcohol. Three more areas on the opposite side of the animal were treated in the same way with the emulsion. In 24 hours the skin of both groins showed slight, patchy erythema and thickening, which increased in the next 24 hours, with the appearance of minute pustules. During the following days the pustules became large, although quite superficial, the skin increased in thickness, and scabs occurred. The maximum reaction was reached in seven days, but quickly subsided, with complete resolution in the next four or five days. Lesions also developed in those areas where the emulsion was applied to the skin and to the wool, the reaction being slighter in the latter case. No lesion developed in the area where the culture was applied to the wool, but a typical lesion, with cupping of the centre of the base, developed where the culture was applied directly to the skin. These experiments were carried out in January, when the weather was hot. The fleece of the animal quickly dried under the warm and dry atmospheric conditions. A more severe and prolonged reaction might be anticipated where atmospheric conditions favour an optimum condition of moisture in the fleece over a longer period.

Cultures and emulsions of the exudate from affected animals have been applied to the uninjured skin of sheep on many occasions, but the results have not differed from those outlined above, so reiteration is unnecessary.

Microscopic examinations of the exudate and pus from the pustules produced in experimental animals have shown the presence of the micro-organism.

Under natural conditions no lesions have been observed on the ventral surface of sheep, and only in one instance have they been found on the limbs. Experiments were made to determine if the skin over certain parts of the body is more resistant to invasion by the micro-organism than that over other parts. A lesion was removed from an affected lamb (sheep L. 2), and emulsified. The emulsion was applied to the skin of the forehead, the skin on the inside of the thigh, and to the skin on the abdominal wall of the same animal, all these areas being free from natural lesions. An experimental lamb was inoculated with the same emulsion in the corresponding areas. The experimental lamb developed the usual transient lesion on the forehead, but none on the thigh or abdomen.

The same result was obtained in the affected animal except that the lesion on the forehead was severe and progressive. The lesion is still active, over three months after inoculation.

A similar experiment was made, using both emulsion and culture. The same affected lamb as used in the previous experiment received an application of emulsion to the skin of the right thigh, and a few inches lower down, to the skin which had been lightly scarified. On the left thigh an area of skin was rubbed with a 24 hours old culture of the micro-organism in broth, and a few inches lower down 0·2 cc. of the same culture was injected subcutaneously. The same inoculations were carried out on an experimental lamb. No reaction was produced, except from the subcutaneous inoculation of culture in the experimental lamb, where a nodular swelling developed in 24 hours, which had slightly decreased in the next 24 hours. There was no breaking down of the tissues and the swelling gradually disappeared. The affected lamb showed in 24 hours a slight induration where the culture had been injected, but it had almost disappeared 48 hours after inoculation. A slight reaction occurred where the skin had been lightly scarified before the application of emulsion, but none in the other areas.

These two experiments indicate that the skin covering the ventral surface of the body and the legs is relatively resistant to invasion by the micro-organism, and, further, that the subcutaneous tissues of an affected animal appear to develop some resistance to the micro-organism. In view of this local immunity of the skin covering the ventral surface of the body and the limbs, the production of lesions on the galbrous skin of the groins may seem to be contradictory and difficult of explanation. The explanation is probably to be found in the fact that the applications of cultures of the micro-organism or emulsion of lesions to the skin of the groins were made after the skin had been thoroughly cleaned with alcohol. This procedure completely removed the coating of yolk, which is usually very thick over the skin of these parts, and with it probably some of the superficial epithelial cells, rendering the epidermis more vulnerable. That this is not a complete explanation is suggested by the failure to produce anything more than a slight reaction in the skin of the thigh after light scarification. It seems reasonable to conclude, therefore, that the thin skin over the groins is less resistant than the thicker skin over the thighs.

The experiments have demonstrated that the severity of the reaction to the invasion of the skin by the micro-organism depends very largely on the individual susceptibility of the animal. Using a susceptible animal (sheep L. 2), a severe lesion was produced by applying an emulsion of a lesion to the uninjured skin of the forehead. This lesion has progressed, and is still active three months after the inoculation. This reaction is more severe than that usually found under natural conditions.

The result of one experiment indicates that the micro-organism probably

grows in the fleece when there is sufficient moisture present, and that the skin may be invaded and a lesion produced under such circumstances.

An experiment was conducted for the purpose of demonstrating if contact of healthy with affected animals would lead to infection of the skin and the production of lesions. Two wethers were placed in the same pen with two affected animals (sheep L. 1 and sheep L. 2) on October 3, 1928, and kept under cover. On January 4, 1929, these two wethers and a lamb were sprayed with water from a hose until the fleece was thoroughly saturated. The hosing was repeated on the following day, and the animals returned to the pen with the affected sheep. The affected ewe from the other stud (sheep M.L. 1), was also placed in the same pen. The animals were examined from time to time, but no lesions could be detected on the experimental sheep. Sheep L. 1 died towards the end of January, and sheep M.L. 1 improved, normal wool pushing the matted areas away from the skin. On June 18, 1929, the experimental animals and sheep M.L. 1 were placed outside under natural conditions. Light showers of rain fell during the day, and again on the 20th. On the 21st more showers developed, with heavy rain in the afternoon. There was little rain after this until July 4, when a heavy shower occurred. July and August were unusually dry months. The sheep were shorn towards the end of August, and no lesions could be found on any part of the body.

The result of this experiment indicates that the disease is not easily conveyed from one animal to another, even under conditions of close contact over a period of ten months.

Antiseptic and Germicidal Action of Various Substances.

The effect of salicylic acid, sodium hyposulphite, and flowers of sulphur in preventing the development of lesions under experimental conditions was investigated.

A merino lamb was taken, and half the staple of wool was removed from an area about 3 inches square. To this area, and to an unclipped area of the same size, a 1 in 1,000 salicylic acid solution in water was applied, saturating the wool. The following day a small section of skin in these two areas was rubbed with the base of a matted mass of wool from an affected animal. Three other areas were inoculated in the same way, one being treated immediately afterwards with a 5% aqueous solution of sodium hyposulphite, another with flowers of sulphur, and the third left as a control. Lesions developed in all the areas inoculated, but the reaction was slightly inhibited by both the sulphur and the sodium hyposulphite.

The inhibiting action of copper sulphate was next tested. A 1 in 1,000 copper sulphate solution in water was swabbed over the skin of the right groin. The skin was lightly dried, and an emulsion of a 48 hours old culture was applied to the surface. The skin of the left groin was treated with the culture as a

control. An area in the right flank was treated with the same copper sulphate solution, and the excess swabbed out of the wool with dry swabs. This area and a control area on the opposite flank were treated with culture. The control areas developed typical reactions, and a few small pustules also developed in the right groin. No reaction developed in the flank treated with the copper sulphate solution. The result of the experiment indicates that sufficient copper sulphate may not be held on the glabrous skin to prevent invasion by the micro-organism, but in a part covered by wool sufficient may be held to prevent the development of the micro-organism and the invasion of the skin. The massive inoculation by the use of a culture of the micro-organism makes this a severe test, so that the results must be regarded as favourable.

In vitro Tests. Tests were carried out by adding 0·1 cc. of a 48 hours old broth culture (broken up by shaking) of the micro-organism to 5 cc. quantities of the substances to be tested in aqueous solution. Subcultures were made after the lapse of five minutes. A 1 in 100 dilution of salicylic acid was found to possess no germicidal properties under the conditions of the test. The action of iodine, copper sulphate, mercuric chloride, and sulphurous acid was determined, and is summarized in the following table:

	Dilution						
	1:1000	1:2000	1:4000	1:8000	1:16000	1:32000	1:64000
Iodine	—	—	—	—	—	+	+
Copper sulphate	—	—	—	—	+	+	+
Mercuric chloride	—	—	—	—	—	—	+
Sulphurous acid (SO_2)	—	—	+	+	+	+	+

— = No growth; + = growth on subculture.

The solution of iodine was made by taking 1 part of iodine, 2 of potassium iodide, and 100 of water.

The sulphurous acid was made by diluting the B.P. preparation, which was taken to contain 5% of SO_2.

The copper sulphate solutions were made from the crystalline salt, so that for the purpose of comparison the dilutions should be $^{25}/_{16}$ of the actual figures given. If this correction is made it will be seen that mercuric chloride is 2·56 and iodine 1·28 times as germicidal as copper sulphate under the conditions of the test.

As copper sulphate is easy to obtain, relatively safe to use, and inexpensive, it should prove suitable for use under field conditions.

Field and experimental observations have indicated that the micro-organism probably grows in the fleece when conditions of moisture are favourable, and that the skin is subsequently invaded. The disease appears to be seasonal, occurring during the winter. It is recommended, therefore, that for the purpose of prevention, young animals should be dipped in a 1 in 500 copper sulphate solution towards the end of autumn, and again a month later, particularly where the disease is troublesome.

The Nature of the Lesion.

In the early stages the lesion is essentially exudative in character. A sero-fibrinous fluid with a moderate number of leucocytes exudes from the surface of the skin, and is absorbed by the overlying wool. This gradually dries, matting the wool fibres together, and forming a dense and tough mass. The exudation continues as long as the lesion remains active, but in the later stages it becomes mixed with a relatively large number of squamous epithelial cells from the surface of the skin. Chronic lesions become less exudative and more keratogenous or desquamative.

In the majority of cases the inflammatory reaction ceases, and the infection is overcome in from one to three months. In these mild cases all the lesions present appear to be of about the same age, and they do not tend to enlarge. In the chronic cases where the lesions may remain active for twelve months or more there is some increase in size of the individual lesion by an extension at the periphery as well as the production of new lesions in other parts of the body. The wool fibre does not appear to become involved, nor is there usually any involvement of the wool follicle as the wool remains firmly attached to the skin. In the later stages there is involvement of the wool follicle, the wool fibre coming away from the skin, particularly in the centre of a lesion. In the chronic lesion the growth of the wool fibre may be arrested, and the whole mass of matted wool come away from the lesion beneath.

A microscopic examination of a section of the skin from a chronic lesion shows the cornified layer to be diffusely invaded by neutrophile polymorpho-nuclear leucocytes. There is vesiculation of the epithelium in the more superficial parts of the *rete mucosum*, the vesicles becoming invaded early by polymorphs. In some parts the epithelial layer may be thin, and vesiculation lead to its almost complete destruction. There may be accumulation of polymorphs about the exit of the wool fibre, but the reaction is not particularly associated with the follicles. There is slight diffuse invasion of the middle layer of the *rete mucosum* by polymorphs, but for the most part the basal cells remain unaffected. In the *cutis vera* beneath the epidermis, and surrounding the follicles, there is a well-developed accumulation of lymphocytes and plasma cells, with an occasional polymorph. Sometimes there are areas of oedema between the follicles, with some haemorrhagic extravasation. The crusts attached to the epithelium are found to consist of keratinized epithelial cells and polymorphs bound together by a sero-fibrinous exudate. In the crusts and in the superficial epithelium are found collections of a branching mycelium. The strands of mycelium are short, and there are many bacillary forms present. Some of the mycelial threads show the development of conidia, which appear to be somewhat compressed vertically. As well as occurring in definite collections, the mycelium, usually in bacillary form, is seen diffusely and irregularly scattered through the superficial parts of the epidermis. In a chronic lesion the *corium* may be invaded, and bacillary

forms are seen sparsely scattered in and around the oedematous areas. For the most part, however, the invasion is confined to the epidermis, and may be purely superficial.

The Micro-organism.

The micro-organism has been cultivated with ease on many occasions, and usually unassociated with other moulds or bacteria. The same micro-organism has been isolated from the lesions of affected sheep from the two studs mentioned above.

Morphology. In the dried exudate from the skin of spontaneous cases of the disease the micro-organism is seen to be present in the form of a branching mycelium, as well as in short, bacillary form. The branching is lateral, the side branches being short and usually somewhat thinner than the main trunk. The mycelium varies in diameter from under 0·5 μ to about 1 μ. With the development of conidia the diameter commonly reaches 1·75 μ. The conidia, which are best studied in stained films of cultures, vary considerably. When stained by Gram's method they are found to vary in diameter from below 0·5 μ up to about 1·75 μ. The bacillary forms vary in length from about 1·8 μ to 4·5 μ, the most common form being 3·6 μ in length. They are often thickened at one end, and somewhat wavy in outline. Sometimes they are bent almost at a right angle at the thickened end, and slightly curved, the general appearance resembling that of a horse-shoe nail. These forms are more frequently seen in cultures. The development of conidia is more abundant in rapidly growing cultures. The main mass of such a culture appears to consist of cocci mixed with some mycelial threads undergoing transformation into conidia, and giving a streptococcal appearance. The mycelium is somewhat thinner at its growing end.

Staining Reactions. The micro-organism stains well with the ordinary aniline dyes, and retains Gram's stain strongly. The staining may be somewhat irregular, particularly in the material from lesions. The conidia also retain the Gram's stain strongly. It is not acid-fast, nor does it retain the fuchsin when decolourization is done with a saturated alcoholic solution of picric acid (Plaut's stain).

Cultivation. It grows readily on peptone media under aerobic conditions, and will also grow in an agar shake at all depths. It grows readily at 37°C., and more slowly, although eventually more luxuriantly, at 22°C. Under conditions of strict anaerobiosis no visible growth occurs.

Peptone Agar. Good growth is obtained in 24 hours at 37°C. A streak culture shows an even, raised, convex growth, somewhat yellowish in colour. Isolated colonies are regular in outline, raised, opaque, somewhat glistening, and convex to pulvinate, and reach a diameter of up to 1 mm. After 48 hours' incubation the colony becomes more irregular in outline, less raised, and more tenacious. Confluent growth shows more irregularities, with piling up of the

growth where inoculation has been heavier. When the agar is moist and growth is rapid, following several subcultures, the confluent growth is usually moist and mucoid, and quite distinct from the dry, tenacious growth of less active cultures. The yellowish colour of the growth is more apparent after 48 hours' incubation. Surface colonies do not tend to spread, nor do they develop a filamentous appearance. Colonies develop well in deep agar, and reach a diameter of from 0·5 to 0·75 mm. in 48 hours. Under the microscope they are seen to be filamentous, with a branching mycelial extension at the periphery. (Figures 7 and 9.) There is no development of aerial hyphae.

At 22°C. growth is slower, and it is usually 72 hours before it is apparent. A streak inoculation gives rise to a raised and wrinkled growth, more massive than that obtained at 37°C. Many lumpy, rounded, elevated masses develop on the surface of an agar slant inoculation, and sometimes the growth is slightly wrinkled. (Figure 4.) At the lower temperature the colour of the growth is more fully developed. This agrees closely in hue with antimony-yellow, but more exactly to hue 16′, tone b (Plate XV, Ridgway, "Colour Standards and Nomenclature"). There is no colour imparted to the agar medium. Surface colonies, instead of taking on the rounded, pulvinate form usually seen in rapidly growing cultures at 37°C., are flatter, with an umbonate centre and raised, lobate-lobulate margins. (Figure 10.)

Glucose Agar. Growth is the same as on peptone agar.

Blood Agar. Deep blood-agar plates show good colony development in 48 hours at 37°C., with a clear zone of haemolysis, 3·5 mm. in diameter, around the colonies.

Loeffler's Blood Serum. Good growth occurs, but is somewhat more tenacious than on peptone agar. There is no evidence of any digestion after 10–14 days' incubation at 37°C.

Gelatin. Growth is not so good as on peptone agar. Liquefaction only occurs when the reaction is adjusted to the alkaline side of neutrality, a pH 7·6 being suitable. A stab culture incubated at 22°C. shows commencing liquefaction in six days. The liquefaction is saccate in type, and develops to a depth of 1 cm. in fourteen days. At 37°C. liquefaction is incomplete at the end of six days and complete in nine days. If equal quantities of milk and gelatin are used liquefaction is more rapid, being complete within six days.

Potato. No visible growth occurs on potato.

Peptone Broth. Good growth occurs at 37°C., with the formation of a pellicle in 24 hours. There is no general clouding of the medium. Sometimes the pellicle is soft, and at other times more tenacious. Growth often occurs at the sides of the tube, producing a frosted appearance on the glass. There is usually a slight sediment, which is more abundant when the pellicle falls. On continued incubation the sediment may become mucoid in character. When growth is less vigorous there is no pellicle formation, and discrete colonies

develop and attach themselves to the sides and bottom of the tube. When inoculation is light, and incubation is at 22°C., a filamentous colony may occur in the depth of the medium.

Litmus Milk. The colour is bleached in four days, and the medium completely digested in from seven to eight days. The characteristic yellow colour is imparted to the medium. At no time is there any definite coagulation. The digestion is from the surface downwards, and the undigested milk at the bottom of the tube appears to be slightly granular in appearance.

Brom-cresol Purple Milk. Digestion is complete in from seven to eight days, with the production of an increasing alkaline reaction. Ammonia appears to be produced, as the addition of permutit to the medium prevents the full development of the colour change of the indicator.

Fermentation Reaction. Glucose and laevulose are fermented in 48 hours, with the production of acid but no gas. Glycerine is fermented more slowly, only a low acidity resulting. No change is produced in maltose, galactose, saccharose, lactose, mannite, dulcite, dextrin, raffinose, arabinose, adonite, inulin, sorbite, salicin, amygdalin, or erythrite. No diastatic action is demonstrable in broth containing 1% of "soluble starch," but the starch is partly hydrolyzed, with the production of erythro-dextrin, but no acid. Urea is fermented with the production of an alkaline reaction.

Nitrate Reduction. No reduction of nitrates can be demonstrated. It has been necessary to carry out the cultivation in a sealed, moist chamber, to prevent the absorption of nitrous fumes from the air.

Indol is not produced.

The Proteolytic Enzyme. There is rapid digestion of casein, but no action has been demonstrated on coagulated blood serum, coagulated egg white, or on fibrin. The ferment is active only in an alkaline medium. It appears to agree with other proteolytic enzymes of bacterial origin in the production of ammonia. No attempt has been made to study this enzyme more completely, but sufficient information has probably been obtained to assist in the identification of the micro-organism.

Pathogenic Action. The micro-organism produces no lesions in rabbits or guinea-pigs when injected intravenously or intraperitoneally. Subcutaneous inoculation leads to local abscess formation. Lesions are produced when an emulsion of the organism is swabbed on the skin after the hair has been pulled out. No lesions are produced if the culture is applied to the uninjured skin, but the pulling out of the hair produces sufficient injury to allow the micro-organism to invade the skin. In 24 hours there is erythema, and the epithelium around the follicles becomes opaque, producing a finely speckled appearance. In 48 hours the skin is thickened, and a sero-fibrinous exudate appears on the surface. The exudate accumulates during the next few days, but the reaction gradually subsides, and resolution occurs in eight to ten days in the guinea-pig and in

fourteen days in the rabbit. The white mouse reacts in much the same way as the rabbit and guinea-pig, but the skin lesions are milder and more chronic.

The skin of the cow and the horse is also susceptible to invasion following superficial scarification. A more severe reaction develops in the horse, the lesion being very desquamative.

Classification and Nomenclature. A consideration of the morphology and cultural characteristics leaves little doubt that the micro-organism belongs to the genus *Actinomyces*. Although the terms Streptothrix and Nocardia have been suggested for many branching, filamentous micro-organisms, the general consensus of opinion is that they should be grouped under the general heading of actinomyces, for the present at least.

The micro-organism appears to be a new species, and the name *Actinomyces dermatonomus* is suggested for it.

DISCUSSION.

The lesion, found in sheep under natural conditions, and produced in sheep and other animals under artificial conditions, is to be classed as a dermatomycosis. It resembles in many respects ringworm, or trichophytosis, of man and the lower animals. One remarkable feature of the disease is the absence of lesions on the more exposed surfaces of the body, except in rare cases, as a secondary manifestation. If the disease were passed on by direct contact one might expect lesions on those parts which naturally are free from wool. To reach the skin on the back and the sides of the animal the micro-organism has to penetrate the thick fleece of wool. It appears from the data collected that the micro-organism probably grows in the fleece when conditions, such as moisture, are favourable. The numerous and scattered lesions suggest that colonies develop in the fleece, and the growth extends between the wool fibres to the skin. The uninjured skin does not offer as favourable conditions for invasion as skin which has been slightly injured or abraded. As moisture favours the growth of micro-organisms in the fleece, it is possible that after rain moisture travelling down the wool fibres to the skin causes some softening of the superficial epithelium which would favour the invasion by the actinomyces. Moisture possibly has a twofold action, therefore, in favouring the growth of the micro-organism and causing superficial softening of the epithelium. As for the most part only young animals are attacked, it would appear that the skin of older animals offers greater resistance to invasion. It is suggested that this species of actinomyces leads a saprophytic existence in the fleece of sheep, and that occasionally conditions become favourable to its invasion of the skin. Although moisture in the fleece is possibly an essential factor in the production of lesions, excessive moisture does not appear to be necessary, as the rainfall during the winter of 1928 was below the average, and the disease was more severe than had been previously experienced.

Biting insects can be excluded as playing any part in the transmission of the disease or the production of lesions in the present investigation.

SUMMARY.

A dermatomycosis, due to a species of *Actinomyces*, affecting young merino sheep, is described.

Although the disease is rare, and only one or two animals may be affected, in some flocks it may on occasion affect over 20% of the young animals.

The cultural characteristics of the causal micro-organism, which has been named *Actinomyces dermatonomus*, are described.

Preventive methods for the control of the disease are suggested.

I am indebted to Professor J. A. FitzHerbert, of the University of Adelaide, for assistance in the choice of the specific name suggested for the micro-organism, and to Dr. Ethel McLennan, Botany Department, the University of Melbourne, for the opinion that the micro-organism belongs to the Actinomycetes.

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DESCRIPTION OF PLATES.

Figure 1. Sheep L. 1, showing well-developed lesions over the skin of the nose, around nostrils, and extending from the corners of the mouth under the chin.

Figure 2. Sheep L. 2, showing the development of lesions about the lips.

Figure 3. A mass of matted wool removed from sheep L. 1. This has apparently resulted from two lesions coalescing which is suggested by the slight constriction in the centre of the mass. Note the deep concave surface at the base of the mass. The mass is photographed in the reverse normal position. Scale in inches.

Figure 4. Culture of *Actinomyces dermatonomus* on peptone agar incubated at 22° C. for 14 days. Note the piled up, bullate, and slightly wrinkled, rugose appearance of the growth. × 1·5.

Figure 5. Microscopic appearance of a stained smear of a rapidly growing 24 hours' old culture of *Actinomyces dermatonomus*. Note the mycelium breaking up into conidia and the relatively large number of conidia present.

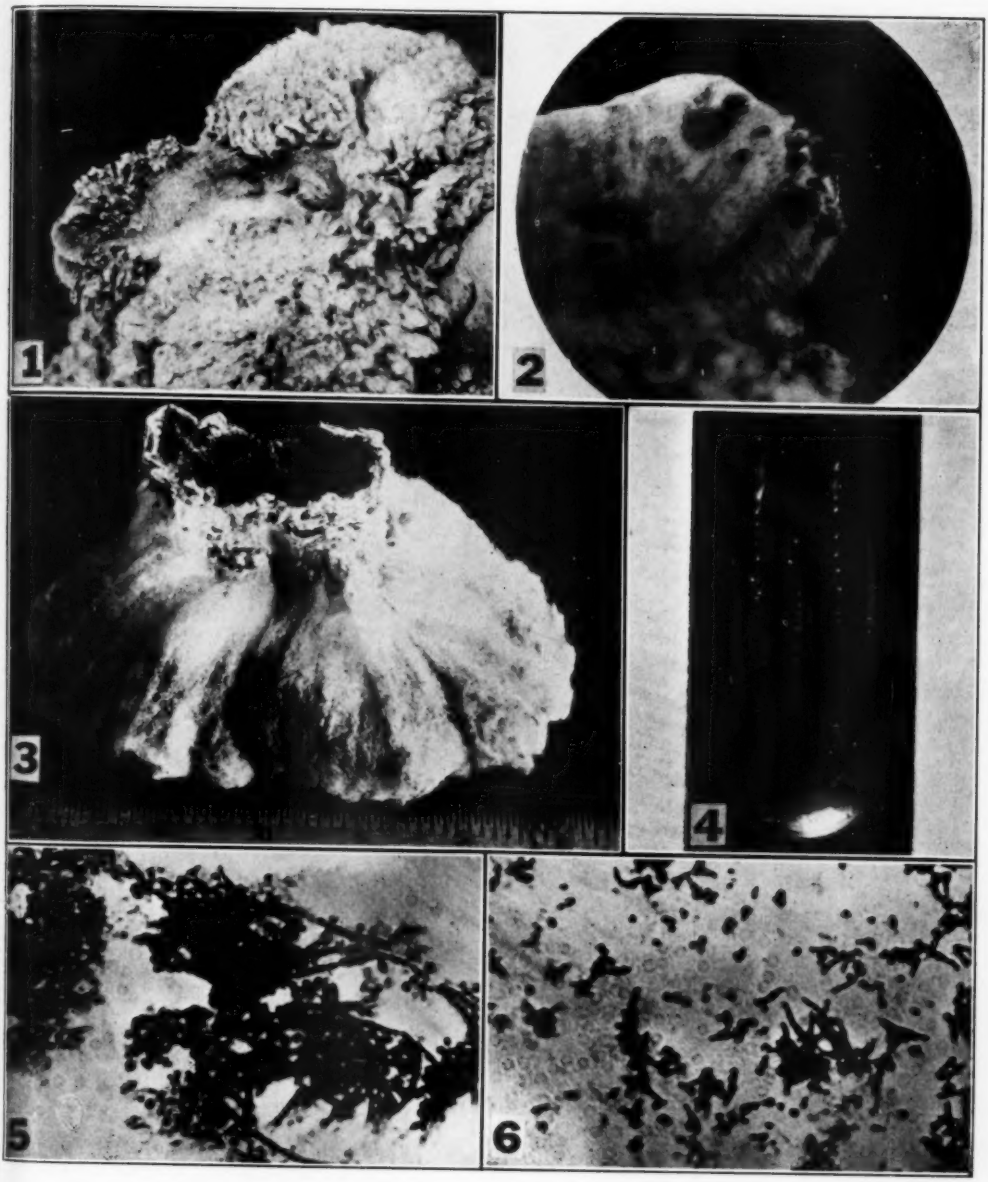
Figure 6. Smear of another young culture of *Actinomyces dermatonomus* showing the presence of bacillary forms and conidia. Many of the bacillary forms are swollen and somewhat bent at one end.

Figure 7. A deep colony of *Actinomyces dermatonomus* in peptone agar showing the filamentous character of the colony. × 94.

Figure 8. Surface colonies of *Actinomyces dermatonomus* on peptone agar. Primary growth. Note the smooth, pulvinate character of colony. × 1·5.

Figure 9. Edge of the same deep colony as shown in Figure 7 under higher magnification showing the branching mycelium. × 180.

Figure 10. Deep and surface colonies of *Actinomyces dermatonomus*. The deep colonies are small, the surface colonies are umbonate with a raised, lobate-lobulate edge. This type of surface colony may occur under conditions of slow development at 22° C. × 1·5.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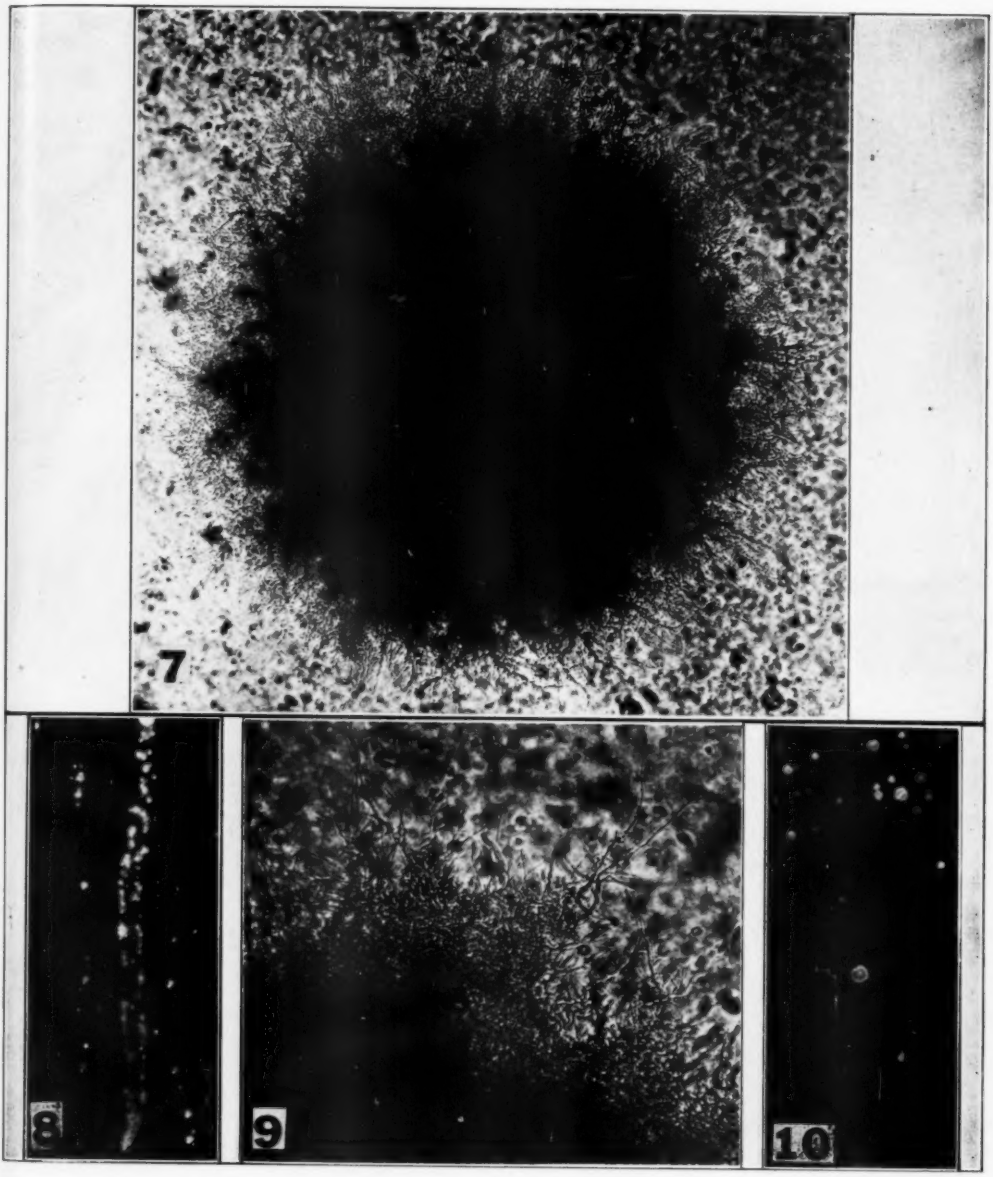
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NOTE ON A METHOD FOR MICRO- KJELDAHL

by

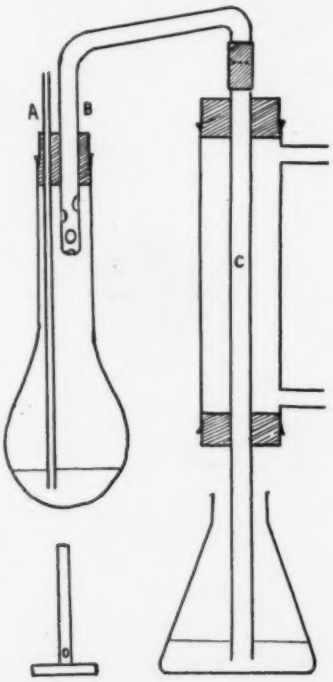
W. J. YOUNG

(From the Biochemical Department of the University of Melbourne).

The following method has been in use for some time in this laboratory, and has proved very convenient, especially for the determination of non-protein nitrogen in blood.

The combustion is carried out in a 100 cc. flask of Jena glass, with a long neck, and of the usual Kjeldahl type.

5 cc. of the protein-free filtrate, obtained by precipitation with trichloracetic acid or with Folin's well-known tungstate method, are employed.



5 cc. of sulphuric acid mixture are added, a small pebble of quartz to prevent bumping, and the mixture is heated by means of a micro-burner until colourless.

The sulphuric acid mixture consists of the following:

50 grammes potassium sulphate.
100 cc. sulphuric acid.
10 cc. saturated copper sulphate.
Water to 500 cc.

The ammonia is distilled over into standard acid by attaching to the apparatus shown in the figure.

The tube, A, passes down into the liquid in the flask.

The splash head, B, is of pyrex glass, of ¼-inch bore, and is made by restricting the bottom opening, and blowing a few holes in the side of the tube, as shown.

C is a tube of cloudy silica, fitted with a condenser about 6 inches in length, and dips down into a 100 cc. Erlenmeyer flask containing 25 cc. of N/100 acid.

Before distilling, 30 cc. of ammonia-free water are added, then 5 cc. of 40% sodium hydroxide are run down the tube, A, by means of a small funnel. This tube is at once connected with an aspirator, and a slow current of air, freed from carbon dioxide and ammonia, blown through. The air is continued throughout the distillation, the flask being heated by a micro-burner.

When the contents are almost at dryness, the heating is discontinued, C is dis nnected at the rubber tube, and is washed down with ammonia-free water.

The acid is titrated in a current of CO_2-free air with N/100 soda, using Wesselow's methyl red methylene blue indicator.

With normal blood the nitrogen corresponds to from 2–4 cc. of N/100 acid, , id the whole determination takes about 30 minutes.

Ed.